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UNIVERSITY OF ALBERTA

FUNCTIONAL RECOVERY AFTER DELAYED PERIPHERAL NERVE REPAIR

BY

MUKAILA AJIBOYE RAJI

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE**

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

SPRING 1994

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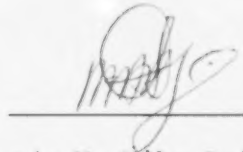

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Tessa Gordon

Dr. Tessa Gordon, PH.D., Supervisor

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TO MY FAMILY

ABSTRACT

To determine the independent contribution of axotomized neuron, denervated distal nerve stump and denervated muscles to poor functional recovery after delayed nerve repair, quantitative electrophysiological and nerve graft techniques were used in a rat model of peripheral nerve injury. In rat hindlimbs, either tibial (TIB) nerve was cut, a common peroneal (CP) nerve sheath denervated or Tibialis Anterior (TA) muscle denervated for periods of up to 12 months prior to repair. Tibial nerve regeneration was initiated by cross-anastomosis of TIB nerves to CP nerve via a contralateral CP autograft. At least 6 months after the nerve repair, the outcome of nerve regeneration was assessed by measuring how many motor nerve fibers make functional nerve-muscle contacts (motor unit number; MUN) and their ability to branch (motor unit force). Functional recovery was further assessed by whole muscle maximal isometric force, muscle weight, total number of muscle fibers, muscle fiber size and distribution pattern of glycogen-depleted motor units.

The result clearly showed that the most adversely affected by delayed nerve repair is the long-term denervated muscle. There is a progressive decline in number of reinnervated motor units with increasing period of muscle denervation. Though the few reinnervating nerve

fibers branch extensively to supply more muscle fibers, the muscle force recovery was further compromised by a decrease in muscle fiber number and incomplete recovery of the muscle fiber size from atrophy.

On the contrary, cross-reinnervated TA muscle recovered fully after prolonged axotomy despite a small but significant fall in motor unit numbers. The force recovery after repair with degenerated graft is quite similar to prolonged axotomy, though there was more severe reduction of reinnervating motor units. However, in the three experimental paradigms, the normal capacity of regenerating nerves to branch and form enlarged motor units was not compromised. This branching can fully compensate for fewer functional motor units if the MUN does not decline below 20% of normal and denervation atrophy is fully reversed. The present study thus shows that the lack of recovery of denervated muscle is the major determinant of poor functional recovery after delayed nerve repair, with lesser but significant role being played by prolonged axotomy and progressive distal stump degeneration. These findings were discussed in relations to possible underlying molecular and cellular mechanisms. Brief abstracts of results from the study have been published (You, Raji & Gordon, 1994; Fu, Raji, Tyreman & Gordon, 1993)

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CHAPTER ONE: INTRODUCTION.

It has long been observed that injured mammalian peripheral nerves have a remarkable ability to regenerate and reinnervate peripheral targets (Ramon Y Cajal, 1928). This regenerative ability is restricted to peripheral axons, and has been attributed to the permissive growth environment of the Schwann cells in the peripheral neural sheaths (Aguayo, 1985). On the contrary, the central nervous system (CNS) neurons regenerate poorly because of the inhibitory milieu of CNS myelin (Schwab, 1990). However, the regeneration process remains poor especially after delayed nerve repair (Sunderland, 1978). Despite a tremendous advance in microsurgical techniques for repairing injured nerves, full restoration of function is quite rare particularly after delayed nerve repair (de Medinaceli, 1989; Sunderland, 1978).

The reasons for poor functional recovery after peripheral nerve injury are not well understood. There are, however, three aspects which are essential for optimal recovery. First, the neurons must survive and synthesize enough materials for sprouting and axonal elongation (Graftstein & McQuarrie, 1978; Ranvier, 1874). Secondly, the distal nerve stump and its nonneural cell population must provide adequate trophic and substrate support for regenerating nerve fibers (Raman Y Cajal,

1928, Holmes & Young, 1942). Finally, the regenerated axons must make functional connections with the appropriate peripheral targets and the target organ must fully recover from denervation atrophy.

When all other factors favor neuronal survival, and axon elongation, the ultimate determinant of functional recovery (the clinical manifestation of regeneration) is the specificity of reinnervation of appropriate target organs (Liuzzi & Tedeschi, 1991).

1.1. FACTORS OPERATING PROXIMAL TO THE LESION.

A key factor in the initiation of regenerative events is the ability of the injured neuron to mount an effective regenerative response following axotomy. The surviving cell bodies undergo a variety of anatomical changes and modifications in gene expression and cellular metabolism (Fawcett & Keynes, 1990) which revert the injured neuron from its transmitting function to a growing mode (Grafstein & McQuarrie, 1978; Gordon, 1983). Precisely what changes occur depend on factors such as age and distance of lesion from the cell body (Grafstein & McQuarrie, 1978; Watson, 1974).

1.1.1. Neuronal Survival following axotomy.

Unfortunately, some neurons die after axotomy.

Although the neuronal death is more severe in young neonatal animals (Schmalbruch, 1984; Sendtner, 1989; Snider & Thanedar, 1989), there is still loss of neurons following peripheral nerve injury in adults (Snider & Thanedar, 1989) especially dorsal root ganglion neurons following sciatic nerve cut.

Several workers have shown that spinal motoneurons (Himes & Tessler, 1989) apparently do not die following sciatic nerve section in adult mammals (Melville et al, 1989; Schmalbruch, 1984), and apparently survive even following long-term separation from targets (Carlson et al, 1979; Gordon et al, 1991; Vanden Noven et al, 1993). However, large numbers of motoneurons in the facial and hypoglossal nuclei of adult rats die following ipsilateral nerve section (Yu, 1988; Arvidsson and Aldskogius, 1982).

It can easily be seen that such loss of neurons, sensory or motor, after axotomy will have a major impact on the extent of functional recovery after peripheral nerve injury. Hence it is important to gain an understanding of the cause of neuronal death and what can be done to prevent it.

The aetiology of neuronal death relates to trophic interactions between neurons and cells in their environment particularly their targets (Oppenheim, 1981; Vrbova et al, 1994). For instance, during prenatal

development, neurons are dependent on their targets for neurotrophic factors (Oppenhein, 1981). This target dependence is still evident in neonatal life when axotomy causes death of many dorsal root ganglion neurons (Himes & Tessler, 1989) and motoneurons (Kuno, 1988; Schmalbruch, 1984). However, for unknown reasons, target dependence declines with maturity and, in the adult, fewer neurons succumb to axotomy (Gordon, 1983; Liuzzi & Tedeschi, 1991).

With regards to preventing death of axotomized neurons, several workers have achieved this by providing various trophic substances. For instance, Rich et al (1987) rescued 100% of the sensory neurons that normally die after sciatic nerve transection by local administration of exogenous nerve growth factor (NGF) to the cut end of the adult rat sciatic nerve. Similarly, Sendtner et al (1990) reported that local application of ciliary neurotrophic factor (CNTF) prevented massive cell death of facial nucleus motoneurons in neonatal rats following facial nerve transection. These data and others suggest that neurons might derive trophic support, including NGF and other survival factors, not only from innervated target (retrograde mechanism), but also from afferent neurons (anterograde influence), axon ensheathing glial cells or even themselves (autocrine mechanism) (Korsching, 1993).

1.1.2. Cell body responses to axotomy.

The main morphological event is "chromatolysis": the dispersal of Nissl substance due to the disintegration of large granular condensations of rough endoplasmic reticulum (Nissl, 1892). The onset of regeneration is associated with expression of new genes and proteins. In general, the proteins produced reflect a metabolic change in the priority of the neuron from the synthesis of neurotransmitter and general maintenance to the re-expression of a more plastic or developmental growth mode (Gordon, 1983).

1.1.2.1. Cytomatrix proteins.

Substances that are abundant in developing axons, such as growth associated proteins (GAPs), tubulin, and actin, have their synthesis enhanced (Skene & Willard, 1981; Tetzlaff et al, 1991; Mathew & Miller, 1993). On the other hand, neurofilament protein, which mainly appears in development when axons have connected with their target and are expanding radially, is decreased. (Hoffman et al, 1987; Oblinger & Lasek, 1988; Tetzlaff et al, 1988). Similarly there is down regulation of neurotransmitter synthesis (Grafstein & McQuarrie, 1978; Gordon, 1983).

Some of the growth associated changes have been directly correlated with physiological changes. Thus

decline in neurofilament protein may account for the decline in nerve fiber diameter (Hoffman et al, 1983, 1988; Dyck et al, 1985) and concomitant increase in latency and decreased amplitude of action potentials on the proximal stump of axotomized nerves (Davis et al, 1978; Gordon & Stein, 1982; Gordon, 1983; Gordon et al., 1991; Jassar et al. 1993). However, some neurons exhibit a paradoxical build up of phosphorylated neurofilament in the soma (whereas normally it is found largely in the axon) (Goldstein et al, 1987, Sinicropi & McIlwan, 1983).

GAP-43 (Neuromodulin) is one type of GAP, whose synthesis is increased 20 to 100 times during the successful regeneration of mammalian peripheral nerves (Skene et al, 1986; Bisby and Tetzlaff, 1992; Tetzlaff et al, 1993) and is specifically concentrated in the tips of extending growth cones. Although its specific function is yet to be determined, it has been postulated that GAP-43 may help establish a productive growth state by regulating the optimal intracellular calcium concentration (Bisby et al., 1988). Liu & Storm (1990) proposed a model of the role of neuromodulin (GAP-43) in neurite growth as follows: Calmodulin in growth cones is bound to neuromodulin. Ligand, such as NGF in case of sensory neurons, binds to receptor and activates the phospholipase C system increasing intracellular free calcium and activating protein kinase C (PKC). PKC

phosphorylates neuromodulin causing it to release calmodulin which can then act on the cytoskeleton and play some role in filopodial function. Calcineurin then dephosphorylates neuromodulin which rebinds calmodulin, thus terminating its action.

1.1.2.2. Axonal transport.

Because the synthetic machinery of the neuron is confined to the cell body and dendrites, the newly synthesized materials for axonal repair must be transported to the growing tip by one of the two rate-components of axonal transport (Grafstein & Forman, 1980). Membranous organelles, lipids and growth-associated proteins are transported by the fast component of axonal transport at a rate of 50 to 500mm/day while the slow component transports structural proteins, glycolytic enzymes and proteins that regulate the polymerization of structural proteins at a rate of 1 to 8mm/day (McQuarrie & Lasek, 1989). However, axonal transport is not crucial for all materials needed for neurite elongation (Vance et al., 1991). For instance, it has recently been shown that axons themselves have the capacity to synthesize major membrane lipids for axonal regeneration (Vance et al., 1994). This finding is in line with rapid onset of regenerative sprouts which can not be accounted for by the relative slowness of axonal

transport(Campenot, 1987).

There are two subcomponents of slow axonal transport: the slower subcomponent (SCa) apparently transport assembled neurofilaments and microfilaments while the faster component (SCb) is hypothesized to transport the cytoskeletal elements required for axonal regeneration (McQuarrie & Lasek, 1989). These cytoskeletal elements which include cytomatrix proteins (actin, clathrin, fodrin, and calmodulin), tubulin, and glycolytic enzymes are transported at a rate of 1 to 8mm/day (Jacob & McQuarrie, 1991). Evidence that the rate of axonal outgrowth closely corresponds with the rate of SCb in many neurons (McQuarrie, 1986; McQuarrie and Jacob, 1991) provides strong support for the "structural hypothesis" of slow axonal transport. Therefore, it is postulated that the rate-limiting factors in axonal outgrowth is the slow component (SCb) of axonal transport (McQuarrie & Lasek, 1989).

1.1.3. Morphological response of the axon.

When axon is completely transected, the axoplasm leaks out of both the proximal and distal segments and the proximal axon undergoes a variable degree of "traumatic" degeneration (Ramon Y Cajal, 1928). Trauma to the nerve may result in cell death or at best, degeneration back to the next node of Ranvier. These

degenerative changes may be secondary to the influx of sodium, calcium ions and a massive loss of potassium and proteins known as "chemical burn" (de Medinaceli & Seaber, 1989).

Within a few hours after axotomy, axons begin to regenerate. The first sprouts in myelinated axons are generally seen coming from the terminal nodes of Ranvier, through the gap left by partial retraction of the Schwann cells (Friede & Bischhausen, 1980; McQuarrie, 1985). Unmyelinated axons sprout equally rapidly (Bray et al, 1972). While these sprouts are forming, the cut tip of the axon swells, inflated with smooth endoplasmic reticulum, mitochondria, and eventually microtubules. If the distal stump is absent, the neurite extensions reach the open end of the transected nerve and a neuroma is formed (Seckel, 1990). The neuroma is formed from regenerating nerve fiber and their associated schwann cell which are surrounded by perineurial ensheathments and embedded in a connective tissue matrix (Thomas,1966).

In the presence of distal stumps, the regenerating sprouts, of which there may be several from each axon grow down the endoneurial tubes. The growth cones are usually in contact with the Schwann cell basal lamina on one side, and with Schwann cell membrane on the other (Nathaniel & Pease, 1963; Scherer & Easter, 1984). Subsequent axonal enlargement and maturation is dependent

on connections with a target, branches that disappear have presumably failed to do so (Aitken, 1949).

The signals at the periphery that regulate transition to regenerative growth operate both locally and in a retrograde fashion (Campenot, 1977; Cragg, 1970). In view of the rapid onset of regenerative sprouting and relative slowness of axonal transport, there must be some local signalling at the axon tips which promote growth cone motility and branching (Fawcett & Keynes, 1990; Campenot, 1987). Growth cone motility and neurite extension in some neurons have been shown to be affected by nerve growth factors, neurotransmitters and electrical events which probably operate locally by modulating the calcium concentration inside the growth cone (Campenot & Draker, 1989; Mattson et al, 1988; Patel & Poo, 1984; Kater et al., 1988). On the other hand, retrograde signalling probably plays a growth supporting and survival role by modulating the expression of regeneration-associated genes (Matthew and Miller, 1990). However, more axons sprouts and regenerating axons grow faster in nerves that have received a conditioning crush previously (McQuarrie, 1985). The enhanced regenerative response following a conditioning lesion may be due to the early availability in the axon of molecules associated with regeneration.

The response of non-neuronal cells in the regeneration of axons across the gap between the severed ends of a transected nerve has been studied using synthetic regeneration chamber (Lundborg et al, 1982; Williams et al., 1983). Such neural entubulation studies have clarified the sequence of events in the regeneration of nerve fibers across a gap, and to isolate factors involved in neurite extension. The outgrowth process begins with the formation of an acellular matrix composed of polymerized fibrin that bridges the gap between the proximal and distal stumps within one week after implantation (Williams et al., 1983). This matrix provides a scaffold for the ingress of macrophages, Schwann cells, endothelial cells and fibroblasts (Williams et al., 1983; Seckel, 1990). These findings suggest that a supportive milieu from non-neural cells and their products must be provided to bridge the gap between the proximal and stumps after nerve transection (Longo et al, 1983; Longo et al., 1984).

With delayed nerve repair and prolonged axotomy,it not clear for how long the axotomized neurons continue to express high levels of growth-associated proteins (Cassar & Tetzlaff, 1991). Similarly, there may alterations in quantity and quality of different cytoskeletal proteins with increasing duration of axotomy. Is up regulation of GAP-43, actin & tubulin maintained in long-term

axotomized nerves? Clearly, answers are needed for these important issues.

1.2. FACTORS OPERATING BELOW THE LEVEL OF THE LESION.

In response to axotomy, the distal segment undergoes Wallerian degeneration (Waller, 1850). There is fragmentation of the axons and myelin disintegration in myelinated axons. Macrophages invade the degenerating distal stump followed by proliferation and movement of Schwann cells, fibroblasts and endothelial cells (Seckel, 1990). The chemical degradation of the myelin and axon fragments is followed by a period of intense phagocytic activity by the infiltrating cells. Finally, the endoneurial tube is emptied of debris and the denervated fiber is then composed of a central core of Schwann cells, the band of Bungner, enclosed in a sheath of endoneurium (Sunderland, 1978). Hence, subsequent axonal elongation through the denervated distal stump is dependent upon a variety of extrinsic molecular cues which are provided by the axons cellular and extracellular environment (Aguayo, 1985; Sanes, 1989 and Martini, 1994). The most important source of such molecular cues for axonal growth is the Schwann cells. These can either express such cues as intrinsic constituents of their cell membrane or secrete them as

extracellular matrix components into the interstitial space (Bunge and Bunge, 1983; Schachner et al, 1990).

1.2.1. Schwann cell.

At the site of axotomy, proliferation and differentiation of Schwann cells occur in response to macrophages invasion and, later on, axonal contact (Weinberg & Spencer, 1975; Jessen & Mirsky, 1992; Aguayo et al., 1976; Bunge et al, 1990). The cellular bridge formed between proximal and distal nerve stump is traversed by regrowing and branching axons, and by migratory Schwann cells (Martini et al., 1990; Bunge et al, 1989). The regrowing axons at the distal stump grow preferentially along the interface between the inner aspects of the Schwann cell basal lamina and the Schwann cell surfaces (Nathaniel & Pease., 1963; Kuffler, 1986). Thus, most of the Schwann cell derived neurite outgrowth promoting activities are strongly expressed at this interface. These include expression of cell surface adhesion molecules: L1/Ng-CAM, N-CAM and its polysialic acid (PSA) components, L2/HNK-1, N-cadherin and integrins (Grumet & Edelman, 1988; Sanes, 1989). Schwann cells also elaborate several basal lamina components including laminin, entactin, type 1V collagen, fibronectin, tenascin and heparan sulphate proteoglycans (HPSG; Sanes,

1989; Brushart et al., 1992; Martini, 1994). The expression of these molecules by Schwann cells is highly modulated by axonal signals and basal lamina components (Bunge et al, 1982; Bunge et al, 1990).

1.2.1.1. Cell surface molecules.

Within six days of nerve injury, there is upregulation of L1/Ng-CAM and N-CAM in the nerve distal stump (Tacke & Martini, 1990). Further, antibodies to L1/Ng-CAM, N-cadherin and integrins, when applied together inhibit virtually all axonal growth on Schwann cells (Bixby et al., 1988; Seilheimer & Schachner, 1988), but none completely prevents growth by itself. These experiments strongly suggest that the Schwann cells-associated molecules are functionally implicated in neurite outgrowth by homophilic binding to their corresponding receptors on neurons (Grumet and Edelman, 1988).

1.2.1.2. Extracellular matrix molecules.

Several studies have provided evidence for the role of components of Schwann cell basal laminal in axonal regeneration (Manthorpe et al., 1983; Sanes, 1989; Wang et al., 1992). In support of such a role, an antibody to

laminin-heparan sulphate proteoglycans is able to inhibit neurite growth both in vitro and in vivo (Wang et al., 1992; Sandrock & Mathew, 1987). Other molecules associated with Schwann cell basal laminae which are upregulated in denervated distal stump include tenascin, PSA and L2/HNK-1 (Daniloff et al., 1989; Zhang. et al., 1992; Brushart, 1988). L2/HNK-1 is particularly interesting because of its exclusive localisation to motor axon-related Schwann cells in denervated nerves (Brushart, 1988), but not in Schwann cells previously associated with sensory axons (Brushart et al., 1992). This characteristic expression pattern of L2/HNK-1 may provide regenerating motor axons preferential outgrowth and pathway specificity over others (Martini, 1994).

After nerve lesion, the Schwann cell basal lamina persist within the endoneurial tubes and maintains the columnar orientation of proliferating Schwann cells to form bands of Bungner (Sunderland, 1952). The regenerating axons and their associated Schwann cells interact with the basal laminal scaffolds (Bunge et al, 1989; Ide et al., 1983) to provide a pathway for regenerating axons and a reservoir of neurite promoting factors (Varon & Bunge, 1978). The observation that recovery of function is greater after a crush injury supports the guidance hypothesis of basal lamina and bands of Bungner since the intact endoneurial tubes guide

the extending growth cones to their original targets. However, with prolonged denervation, the Schwann cell basal lamina fragments and disperse within the endoneurial tube (Gianini and Dyck, 1990). This progressive breakup and dispersion of Schwann cell basement membrane (SCBM) with time after transection may lead to faulty neurite outgrowth guidance and target finding.

Another change in the distal stump which adversely affect regeneration outcome is collagenization of denervated endonurial tubes with delayed reinnervation (Sunderland, 1978). This leads to a reduction in size of the tubes by a factor of up to 80% (Sunderland, 1950). Such endoneurial tubes with reduced lumen may constitute a considerable physical resistance to regenerating axons.

1.2.1.3. Neurotrophic factors.

There is extensive evidence that soluble diffusible neurotrophic factors are released from distal regions of injured nerve, and that such factors subsequently stimulate axon regeneration (Liuzzi & Tedeschi, 1991; Korsching, 1993). After injury, Schwann cells synthesize nerve growth factor (NGF), brain derived neurotrophic factor (BDNF; Richardson & Ebendal, 1982; Heumann et al., 1987; Acheson et al., 1991), platelet derived growth

factor (PDGF; Raivich & Kreutzberg, 1987) and insulin-like growth factors (IGF-I & II; Kanje et al, 1989; Hansson et al., 1986). Such activated Schwann cells also express epidermal growth factor receptors (EGFR; Toma et al, 1992) and low affinity NGF receptor (LNGFR; Taniuchi et al., 1988; Raivich et al., 1991). The exact role of the presence of the LNGFR on Schwann cells remains speculative but the following function has been proposed. When NGF binds to its receptor on Schwann cells, the ligand-receptor complex is not internalized (Taniuchi et al, 1988), and thus NGF may serve as an elongation factor for regenerating axons by binding simultaneously to NGF receptors on axons and Schwann cells. Several studies support this concept that substrate bound NGF promotes regeneration (Sandrock & Mathew, 1987; Gunderson, 1985). Also the NGF bound to NGFR on Schwann cell may serve as a local source for elongating axons before contact with target is re-established during regeneration. Nerve sheath NGF is taken up by the growing axons, thus enhancing regeneration of injured neurons (Brown et al., 1991). It is also possible that NGF is trophic to the Schwann cells themselves in an autocrine manner (Korsching, 1993).

The observation that NGF receptor expression by Schwann cells decreases considerably in the distal stump after 10 weeks of denervation (Taniuchi, 1988; You et

al., 1994) bears relevance as a limiting factor in nerve regeneration after a delay. The reduction in NGF receptor may be due to a reduction in the number of Schwann cells or a decrease in NGF receptor density per cell with increasing degeneration of distal stump.

On the whole, these data suggest that NGF and other neurotrophins continue to serve as a neurite promoting factor in adult PNS, and that the upregulation of these factors in distal nerve stumps following nerve injury serves to facilitate the outgrowth of daughter axons. It must be mentioned, however, that some molecules such as ciliary neurotrophic factor (CNTF) and myelin-associated protein (P0) are down-regulated in the denervated peripheral stump (Seniuk et al., 1992; Leblanc & Poduslo, 1990).

1.2.2. Role of macrophages.

Several studies have shed more light on close association of macrophages with success of regeneration in the peripheral nervous system (Perry et al, 1987; Lunn et al., 1989; Brown et al., 1991).These studies typically use cell-impermeable entubulation or C57BL/Ola mice that have sparse macrophage invasion. In these situations, Schwann cells do not proliferate, myelin persists and degeneration is subsequently delayed (Beuche and Friede, 1984); and regeneration is impaired.

These observations support the role of macrophages in removal of myelin debris in degenerating portions of injured nerve probably along with the participation of Schwann cells (Williams & Hall, 1971; Sunderland, 1991). Macrophages may also participate in redistribution of lipid from degenerating axons to regenerating axons (Perry & Gordon, 1988). For instance, Ignatius and co-workers (1986) show that macrophages increase their expression of apolipoprotein E (Apo-E). This Apo-E may bind to LDL-receptors on sprouting neurites & Schwann cells thus transporting accumulated lipids from one cell to another and providing some trophic support for axonal sprouting (Ignatius et al, 1986).

Further, the observation in vivo that macrophage recruitment in the first 3 to 5 days post axotomy temporally coincides with Schwann cell proliferation (Perry et al., 1987; Clemence et al., 1988) provides further support for stimulation of Schwann cell division by macrophages. How that happens still remains unclear. One suggestion is that macrophages which have digested myelin membrane fragments release a soluble Schwann cell mitogen (Baichwal et al, 1988). Besides, a surface component of the axon membrane also provide a crucial mitogenic signal when in contact with Schwann cells (Bunge et al, 1990; Bunge, 1988).

Moreover, there is evidence that macrophages recruited in the degenerating regions of injured nerve induced the increased synthesis of NGF in Schwann cells (Heumann et al., 1987) by secreting the stimulatory agent interleukin-1 (Lindholm et al., 1987; Guenard et al, 1991). However, NGF receptor synthesis in Schwann cells is downregulated by axonal contact (Heumann et al., 1987).

It is clearly important to know the fate of the non-neuronal cells in long-term denervated distal stump. Do they continue to produce the growth-supportive factors such as cell adhesion molecules, trophic factors and extracellular matrix proteins with prolonged distal stump degeneration ? It is therefore of great theoretical and practical importance to investigate the capacity of long-term denervated stump to support nerve regeneration.

1.3. ACCURACY OF REGENERATION.

The major problem of peripheral nerve regeneration involves the specificity of reinnervation. This specificity involves: (1) specificity at the level of the nerve i.e, axonal pathfinding or the ability of axons to find the appropriate path leading back to their targets; and (b) specificity at the level of the target (Liuzzi and Tedeschi, 1991).

1.3.1. Specificity at the level of distal stump.

It is well known that nerve fibers regenerate more accurately following crush rather than cut injuries (Sunderland, 1978). The difference is because the endoneurial tubes and Schwann cell basal lamina are left intact in the case of crush injures (Haftek & Thomas, 1968); and regenerating axons usually remain in their parent tubes and are guided back to their targets. By contrast, in the transection injury, even with the intervention of the most advanced microsurgical repair, the disrupted tubes result in axonal sprouts entering inappropriate tubes in the distal stumps leading to inappropriate targets. However, the accuracy of regeneration following cut injuries varies according to body region, age, species, composition of nerve fiber types, repair technique and degenerative changes in distal stump and target.

The occurrence of aberrant innervation after axotomy is well established in the neuromuscular system of adult mammals (Brushart & Mesulam, 1980; Sperry, 1945). This is in contrast to the results following transection of motor axons in neonatal rats (Aldskogius & Thomander, 1986) where regeneration can restore appropriate neuromuscular connections in the periphery. Also, a degree of positional selectivity has also been shown in the reinnervation of the adult rat diaphragm and serratus

anterior muscle (Laskowski & Sanes, 1988).

The mechanisms for the restoration of original innervation in these examples are not clear. There are at least two broad possibilities. First, specificity could originate in the target muscle cells which could provide diffusible and/ or surface-bound cues for motor axons (Fawcett & Keynes, 1990). A second possibility is that Schwann cells or fibroblasts might provide guidance cues for axons (Wingston & Donohue, 1988).

There is increasing evidence that regenerating rat peripheral axons can to some extent identify and direct their growth into appropriate branches of nerve trunk (Politis, 1985; Brushart, 1988). This raises the possibility that specific axon guidance cues operative during embryonic development might function to a limited extent during later regeneration (Fawcett & Keynes, 1990). Several studies support this possibility. For instance, Politis and colleagues, using a Y-tube choice paradigm, found that regenerating axons in adult rat sciatic nerve will choose viable distal stumps over non-viable nerve stumps. Further experiments by Politis showed evidence of axonal sorting at branch points of adult peripheral nerves (Politis, 1985). He found that axons in the proximal stumps of the peroneal nerve consistently chose the distal stump of the peroneal nerve over that of tibial nerve. Brushart and Seiler (1987)

also found that motor axons preferentially chose the motor stump over the sensory stump, when the two choices are provided. This finding is further supported by the selective ability of previously motor axon-associated Schwann cell to re-express L2/HNKI molecules that specifically enhance regenerating motor axons (Brushart et al, 1992; Martini et al., 1992).

The Politis studies and those of Brushart and Seiler suggest firmly that in adults, the choices made by axons at the level of the nerve can be determined by local guiding cues. It appears, however, that mechanical factors within the nerve can override the effects of these cues and result in axonal misrouting into inappropriate targets (Liuzzi & Tedeschi,1991).

1.3.2. Specificity at the level of the target.

Another issue of clinical significance is whether axons that have regenerated to the periphery can identify appropriate types of target organ. A few studies provide evidence for some degree of target selectivity. For example, Horch (1988) showed that sensory receptor preferentially reinnervated the same receptor types as they had innervated before nerve injury. Similarly, Munson and colleagues (1988) have reported a high specificity of reinnervation of tendon organs by muscle

afferents after transection and resuture of the nerve to medial gastrocnemius in adult cats. Moreover, there is some evidence for a localized neurotropic influence exerted by molecules in the muscle basal lamina. This was demonstrated by the fact that, even in the absence of living muscle cells, regenerating motor axons grow back to the sites of former neuromuscular junction on the empty basal lamina sheaths (Sanes et al, 1978). Several molecules which have been shown to regulate acetylcholine receptors on muscle surface may be neurotropic to the elongating axons (Frank & Fishbach, 1979; Usdin & Fishbach, 1986; MacMahan & Wallace, 1989). These molecules such as agrin, ARIA (acetylcholine receptor inducing activity) and calcitonin gene-related peptide (CGRP) are released by motor nerve terminals and localized in the synaptic basal lamina (MacMahan & Wallace, 1989). How these factors affect the specificity of muscle reinnervation requires further research. Such factors may then be exploited to improve precision of target reinnervation following peripheral nerve injury.

Despite the above findings, regenerating motor axons fail to show specificity at the level of the muscles or muscle fibers. For instance, axons that normally innervate slow-twitch muscle fibers show no preference for these fibers and when misrouted, innervate fast-twitch muscle fibers (Gillespie et al., 1987; Gillespie

et al., 1986; Gordon, 1988;). Clinically, functional recovery is very uncommon especially after cranial nerve lesions and following surgical repair of proximal plexus injuries, in spite of reasonable numbers of regenerating axons, because of aberrant innervation (Milesi, 1988; Gordon, 1994). The axonal misrouting leads to deranged central reflex modulation and disturbed orderly recruitment of motor units according to the size principle (Thomas et al, 1987; Sumner, 1990). Unfortunately, central reeducation or adaptability to misdirected regeneration does not occur to any appreciable extent. (Sumner, 1990).

1.4. FACTORS OPERATING AT THE LEVEL OF DENERVATED TARGET.

Due to progressive atrophic changes in denervated muscle, it might be expected that the process of its reinnervation would vary according to time which elapses before new nerve fibers return (Gutmann and Young, 1944). Following denervation, there is a rapid initial loss of 30% muscle weight by the end of first month. This loss increases to 50% by the second month beyond which the process then slows to a relatively stable state at about the fourth month. (Sunderland, 1991). From this time onwards the weight loss varies between 60 and 80%. There is concomitant reduction of cross-sectional area of fiber

which is 70% by the end of second month. (Sunderland, 1991). Clearly, there is more impact on individual fiber size than the whole muscle weight because of increased amount of connective tissue compensating for the atrophic fibers. With increasing duration of denervation, some fibers continue to degenerate and eventually disappear (Gutmann and Young, 1944; Sunderland, 1978).

These gross changes in the extrafusal fibers are paralleled by disorganization of endplates and upregulation of extrajunctional acetylcholine receptors (Miledi, 1960; Bambrick and Gordon, 1987). However the basal lamina components and acetycholine receptors of the endplate are remarkably stable after prolonged denervation and even in the absence of receptive muscle (Sanes et al., 1978). These endplate sites still accounted, at least in part, for the attraction of regenerating nerves with subsequent synapse formation (Gutmann and Young, 1944; Kuffler, 1986).

For successful axonal regeneration in denervated muscle and ultimate functional synapse formation, the growth environment provided by the muscle basal lamina, extracellular matrix and non-muscle cells must be maintained for a long time. After denervation, Schwann cells and fibroblasts at the endplate region and within the intramuscular nerve sheaths migrate laterally (Reynolds and Woolf, 1992) and undergo similar changes in

gene expression to those in the nerve trunks (Taniuchi et al., 1998). However the spatial distribution of these changes may depend on the nature of the lesion (Lunn et al., 1990). Similarly there is upregulation of cell adhesion molecules (N-CAMS), fibronectin, tenascin and HPSG and they accumulate at denervated endplate regions of the muscle (Gatchalian et al., 1989). These have been attributed to the intramuscular fibroblasts (Sanes et al., 1986) and these molecules have been linked with the preference of regenerating nerves to reinnervate the endplate (Sanes et al., 1978). Moreover, the branching of nerve fibers is adequately promoted possibly via the actions and modulations of L1, N-CAM and PSA (Landmesser et al, 1990). For example, Landmesser et al., (1990) showed that branching occurs during a period, in development, when the PSA content of the nerves is high, a situation which would also favour branching by reducing the adhesive forces between axons mediated by cell adhesion molecules. While the adhesive surfaces on which regenerating nerves grow and branch are not the same as developing nerves, they may share common mechanisms with immature nerves and muscles for promoting and controlling nerve branching (Booth et al, 1990).

Ultimately, efficiency of reinnervated muscles will depend on complete maturation of restored axon pathways, reconnection with appropriate end-organs and full

recovery of muscle fibers from atrophy and intramuscular fibrosis. It is not however clear whether the non-muscle cells i.e Schwann cells and fibroblasts in the intramuscular sheaths and endplate regions of longterm denervated muscles continue to support adequate nerve growth and functional nerve-muscle connection. Do fibroblasts at the denervated endplates continue to express high levels of CAMS and ECM molecules? Success of nerve branching and appropriate target reconnection may simply be contingent on quality and quantity of these non-muscle cells.

1.5. PURPOSE OF STUDY

Regeneration and functional recovery after delayed repair could be compromised by: (a) decay of the growth response of the neuron with time and/or (b) a decline in the ability of distal stump to support regeneration and/or (c) reduced capacity of denervated muscle to accept reinnervation and recover from denervation atrophy. There are therefore three anatomical components which influence regenerative outcome: neuron, nerve and muscle. However, the relative and independent contribution of each of these components to failure of regeneration is not clear.

The purpose of this work was to systemically investigate which of the three components are the most detrimentally affected by delay, using electrophysiolocal and nerve-graft techniques in a rat model of peripheral nerve injury.

CHAPTER TWO: METHODS

2.1. SURGERY AND PREPARATION.

All the surgical operations were performed on 143 adult (300 400g) female Sprague-Dawley rats under sodium pentobarbital anaesthesia (60mg/kg) and sterile condition. In rat hindlimbs, either (TIB) neurons were axotomized, a common peroneal (CP) nerve sheath denervated or Tibialis Anterior (TA) muscle denervated for periods of up to 12 months before tibial nerve regeneration was initiated by nerve graft cross-union using 8-0 silk suture (Ethicon Ltd, Peterborough). Nerve regeneration and muscle reinnervation were prevented by sewing the cut proximal nerve stumps onto neighbouring innervated muscles. This procedure effectively prevented regeneration (Watson, 1970). Careful attention was made to ensure the integrity of the blood supply of the operated hindlimbs.

2.1.1. Prolonged neuron axotomy prior to the nerve regeneration.

Regeneration of the chronically axotomized TIB neurons was initiated by detaching the proximal nerve stump from innervated muscle and suturing it to a fresh CP nerve graft (10-15mm from the opposite leg). The graft

was in turn sutured to the distal stump of freshly cut CP nerve very close to the entry into the anterior compartment containing the TA muscle. Thus long-term axotomized neurons regenerated through a freshly denervated CP nerve stump (fresh autograft) to a freshly denervated TA muscle (figure 1A).

2.1.2: Degeneration of distal nerve sheath prior to nerve repair.

The chronically denervated CP nerve segment was used, in the opposite leg, to bridge freshly cut TIB nerve to freshly cut CP nerve to direct nerves to freshly denervated muscle (figure 1B).

2.1.3: Prolongation of muscle denervation prior to regeneration.

CP nerve was cut at the knee and later freshly cut TIB nerve was directed through a fresh CP autograft to innervate the chronically denervated muscle (figure 1C).

2.2. PREPARATION FOR ACUTE EXPERIMENTS.

Final acute experiments were carried out 6-12 months after the nerve-graft cross-union. Prior to the acute experiments, all rats were glucose loaded for 3-5 days with the use of 5% dextrose in the drinking water to

increase muscle glycogen storage. Same age and weight-matched rats that had no prior surgery were used for control data measurements.

Pentobarbital sodium (45mg/kg 1p) was administered for general anesthesia followed by 0.1mg/kg IP atropine sulphate to reduce tracheal secretions. The trachea was cannulated for mechanical ventilation if necessary. Maintenance doses of anesthetic diluted in a 5% dextrose saline were administered every 1-2 hour via a cannula in the right external jugular vein. Depth of anesthesia was monitored by recording electrocardiogram (EKG) activity from needle electrodes, which were inserted subcutaneously in the two arms. Any generalized muscle activity in the lightly anesthetized animal was readily detected on the oscilloscope or audiomonitor and corrected by administering anesthetic. To maintain blood volume, 0.5-1ml of the 5% dextrose-saline solution was administered via the intravenous cannula approximately once per hour throughout the experimental procedure.

A laminectomy from T12 to L6 was performed, and the TA muscle in the operated hindlimb was isolated unilaterally by denervating all hip, tail, and limb muscles except the TA (figure 2A). The distal tendon of the TA muscle was freed along with a small piece of calcaneous bone around its point of insertion and securely tied with 2-0 silk suture for force recording.

A surface bipolar patch electrode was sewn onto the muscle fascia for direct muscle stimulation or electromyographic (EMG) recording.

The bared ends (~5mm) of two Teflon-coated fine silver wires (75um), inserted into the adductor femoris and semimembranous muscles ~10mm apart longitudinally on either side of the sciatic nerve, were used to stimulate the sciatic nerve. The wire was threaded into a 27-gauge needle, and the bared end was coiled around the needle. Intramuscular insertion of the needle left a coil of bare wire that was very stable and highly effective as stimulating electrode.

Both hindlimbs were immobilized at the knees and ankles, and the distal TA tendon of the prepared leg was secured to a Grass force transducer (FTO3 with springs adjusted for 10 and 0.05 N maximum force range for whole-muscle and MU recording, respectively). Care was taken to maintain the in vivo position of the muscle. A mineral oil pool was made around the spinal cord by extending the skin flaps on the back of the animal. Temperature was monitored from the lower hindlimb muscles, the spinal cord, and the rectum with temperature probes (Yellow Springs Instrument) and maintained between 35 and 37 ^{0}C by radiant heat above and a heating pad underneath the animal. Ventral roots L4, L5 and L6 were exposed by cutting the dura mater longitudinally, isolated by the

use of gentle suction and cut as they entered the cord.

Single TA MUs were functionally isolated by stimulating fine filaments dissected from one of the three ventral roots L4, L5, or L6. A second criterion for unit isolation required no change in force, or EMG amplitude and shape.

2.3. FORCE AND MOTOR UNIT FORCE RECORDING.

Force and EMG signals were amplified, viewed on a Tektronix dual time base storage oscilloscope (model 5441), monitored continuously on a Gould 1200S pen recorder, and digitized and stored on disk by an LS1-11 computer (Digital Equipment). Maximal muscle tetanic and twitch forces were recorded under optimal muscle length in response to suprathreshold sciatic nerve stimulation. Ventral roots were teased into small filaments which contained only 3-10 motor nerve fibers innervating the TA muscle. Ventral root filaments were stimulated with gradually increasing stimuli to progressively recruit single MUs judged by all-or-none increments in twitch force and associated unique EMG signals (Fladby and Jansen, 1990; McComas, 1991; Stein and Yang, 1991). All-or-none firing at threshold stimulation was carefully checked over several minutes to ensure that single unit was isolated. Twitch forces of single MUs were obtained

by digital subtraction. The twitch forces from at least 30-40% of the total MU population were measured to obtain a representative sample (figure 2B). Muscle-unit force and EMG potentials were recorded from isolated single MUs in the TA muscles as a measure of muscle-unit size. The mean MU twitch force was calculated. The number of MUs in the reinnervated TA muscles was obtained by dividing the muscle twitch force by the calculated mean MU twitch force (figure 2C).

2.4. SINGLE MOTOR UNIT ISOLATION AND GLYCOGEN DEPLETION.

At the end of muscle and MU force recordings, a single MU from control and reinnervated muscle was isolated for further physiological characterization and subsequent glycogen depletion. Classification of MUs into fast fatigable (FF), fast fatigue resistant (FR), fast fatigue intermediate (FI), and slow (S) was based on contractile speed, "sag" and fatigability. The criteria of isolation of a single unit was based an all-or-none twitch contraction and EMG response. A motor unit was selected only if the threshold voltage was lower than 60 volts and at least 10 times less than the threshold for any other MUs associated with the same filament. These criteria were devised in order to keep the MU for 1-4 hours of recording required for adequate depletion of the

glycogen in the muscle fibers of the MU (Totosy de Zepetnek et al.,1992). Parameters measured in isolated units included 1) twitch force , contraction time (CT), time to peak contraction (TTP), half-rise time (HRT), and half-fall time (HFT) during a single contraction; 2) maximum force produced during fused tetanic contraction;3) "sag": the presence or absence of a drop in the force during unfused tetanic contraction;4) fatigability: the relative decline in the force produced during a two-minute tetanic stimulation (trains of stimuli at 40 Hz with intervals of 1 second). Finally one single MU from each muscle was repetitively stimulated (starting with 1 Hz) with trains of tetanic stimulation consisting of five pulses at 50 to 100 Hz to deplete the glycogen store in the muscle unit fibers for later recognition, fiber typing, and morphometric analysis of number and area of fibers on muscle cross sections.

2.5. HISTOLOGICAL PREPARATION AND HISTOCHEMISTRY.

At the end of glycogen depletion, the TA muscle from both legs were very quickly excised and placed on saline-soaked gauze squares on ice. Muscles were weighed, cut into three blocks, and each block was mounted with OCT (ornithine carbamy-transferase) compound onto a cork, frozen in a pool of melted isopentane cooled with liquid

nitrogen (-160^{0}C), and immediately placed in a freezer (-75^{0}C) for storage. Serial cross sections, 10um thick, were later cut from each muscle block in a cryostat at -20^{0}C. Glycogen-depleted muscle fibers belonging to the fatigued MU were identified and enumerated by the use of periodic acid-Schiff (PAS) staining (Pearse 1960) and typed as slow oxidative (SO), fast oxidative-glycolytic (FOG), fast intermediate (Fint), or fast glycotic (FG) by the use of myofibrillar ATPase (mATPase)with acid preincubation modified from Brooke and Kaiser (1970), myofibrillar ATPase with alkaline preincubation modified from Guth and Samaha (1970), and in some cases, nicotinamide adenine dinucleotide diaphorase (NADH) according to Dubowitz and Brooke (1973).

2.6. MUSCLE FIBER MEASUREMENT.

The number of glycogen depleted muscle fibers or the muscle fibers in single motor units was directly counted in the section containing the largest number of glycogen-depleted fibers. The section containing the greatest number of fibers was also used to measure whole muscle and muscle fiber CSAs. The total muscle cross-sectional area was measured directly using a microcomputer digitizing software program (JAVA, Jandel Scientific). Muscle fiber density within a constant field of 0.63mm^{2} was determined for six fields in different regions from

normal and reinnervated TA muscles. The average density was expressed per millimeter squared and converted to total number of muscle fibers by multiplying this fiber density by the total muscle CSA. The total number of muscle fibers in the muscle was obtained by multiplying the mean fiber density by the total muscle cross-sectional area.

The size of glycogen-depleted fibers from single MUs was directly measured. Mean muscle fiber size was acquired indirectly by dividing the total muscle cross-sectional area by total number of muscle fibers.

2.7. STATISTICS AND DATA ANALYSIS.

Standard non-parametric statistical tests were used to compare the results from various experimental groups. All data were presented as arithmetic mean ± standard errors (SE). Statistical significance was accepted for $p < 0.05$. Comparisons of immediate repair were made with previous data collected in our laboratory from direct nerve-nerve suture (no graft) surgical paradigm by Dr Susan Fu.

CHAPTER THREE: RESULTS.

A total of 143 rats were used from which force recordings and histological measurements were made. In considering the possible effects of the use of two sutures in our nerve-graft paradigm, we first compared the recovery outcome in reinnervated TA muscle after immediate nerve-graft suture and immediate nerve-nerve suture with normal unoperated TA muscles. From figure 3, it can be seen that the muscle force and motor unit number (MUN) recovery were not significantly different between the three groups. Hence, we know that the presence of an extra suture and 10 - 15mm long autograft was not having a major effect on recovery as previously shown by others (Pover & Lisney, 1989; Zhao et al, 1992). However the extra suture resulted in considerably more variability between animals.

3.1. PROLONGED AXOTOMY.

As illustrated in figure 4(A), the muscle force recovery was very well maintained even after 6 months of delayed repair of axotomized motoneurons. The mean whole muscle twitch force was 1913 ± 115mN after immediate repair (IMR; N=6) and 1987 ± 69.6mN after six months of delayed repair (DLR; N=4). There was a small but insignificant increase in motor unit size (20.5 ± 3.9mN)

with no delay and 28 ± 6.3mN at six months delay (figure 4B) which was associated with a progressive and just significant fall in motor unit number (110 ± 20.1 in IMR and 77 ± 17.5 in DLR). The mean motor unit force observed represented all the muscle units as depicted in figure (5) which shows the normal distribution and greater than 100 fold range in unit twitch force in normal and reinnervated TA muscles. The reinnervated unit population distribution was shifted slightly but not significantly to the right as a result of increased motor unit size which accompanied the decrease in motor unit numbers. Motor unit force is largely a product of muscle fibers in the unit (motor unit size) and the unit fiber size. The motor unit force measurements were made independent of fiber size. One would predict that if fiber size remained the same, muscle CSA and muscle fiber number would be normal.

It is evident from figure (6) and (7) that after 6 months of axotomy before repair that muscle recovered its former CSA (26.37 ± 1.91mm^2 in IMR and 29.9 ± 1.45mm^2 in 6 month-DLR). To see if all fibers are reinnervated, muscle fiber numbers was computed by multiplying the measured fiber density by whole muscle CSA. As shown in figure 6(C), there was no significant change in muscle fiber number whether a cut nerve was freshly repaired or delayed (9580.8 ± 514.5 in IMR and

9529 ± 764.2 after 6 months of DLR). Average muscle fiber CSA was indirectly derived by dividing whole muscle CSA by whole muscle fiber number. There was no significant difference in mean fiber CSA between IMR (2752.3 ± 269.4 uM^2) and DLR (3137.7 ± 526.89uM^2).

Although the CSA remained the same after delayed repair, spatial distribution of muscle fibers in a single motor unit from a normal unoperated rat (figure 7A) and reinnervated (figure B & C) glycogen-depleted muscle was different. In normal muscle, glycogen-depleted muscle unit fibers were intermingled with nonunit fibers in a mosaic distribution (figure 7A) in contrast to the clumped pattern seen in reinnervated muscle units (figure 7 B & C).

3.2. GRAFT DEGENERATION.

The progressively deteriorating growth environment of denervated sheath can still support enough regenerating motoneurons to produce good force recovery as shown in figure (8A). Whole muscle twitch force was 1913 ± 115mN with fresh graft repair (FGR) and 1741 ± 20.86 with 6 month old graft repair (OGR) and 1636 ± 402.4 mN in 12 month OGR. However, there was a dramatic increase in motor unit size up to 4-5 times normal (20.5 ± 3.91 mN in FGR, 99 ± 18.87 mN in 6 month OGR and 33.2 ± 1.02 in 12 month OGR; figure 8B). Concomitantly, the

motor unit number supported by the nerve graft fell precipitously up to about 25% of the normal with increasing age of graft (110 ± 20.05 in FGR, 25 ± 6.12 in 6 month OGR; figure 8C). This fall in growth supporting capacity of degenerated graft is in line with previous morphological observations of progressive fragmentation of Schwann cell basal lamina in denervated distal stump (Gianini and Dyck, 1990; Salonen et al, 1987). Generally, the process of compensatory enlargement of reinnervated motor unit had a high variability as demonstrated by the large standard error of the mean. Despite this, the distribution of the motor units population was normal but significantly shifted to the right (figure 9) indicating an increase in size of all motor units which largely compensated for drastic reduction in regenerating and reinnervating motor axons.

Despite over 70% reduction in regenerating neurons which made functional nerve-muscle connection, the few that reached the muscle were able to sprout voluminously and reinnervate all the available muscle fibers. Figures 10 and 11 showed no significant change in mean whole muscle CSA (26.37 ± 1.91 mm^2 in FGR, 27.4 ± 2.33 mm^2 in 6 month OGR and 28.59 ± 1.38 mm^2 in 12 month OGR) ; muscle fiber number (9580.8 ± 514.5 in FGR , 9993 ± 299 in 6-month OGR and 9277.7 ± 697.6 in 12-month OGR); and mean muscle fiber CSA (2752.6 ± 269.4 uM^2 in FGR, 2742 ±

128.3 uM^2 in 6-month OGR and 3081.5 ± 505.5 uM^2 in 12 month OGR) whether a fresh or 1 year old graft was used. Apparently the neuronal capacity to branch intramuscularly was optimally supported by the muscle environment and not compromised by having grown through a predegenerated graft.

There was similar tendency as in prolonged axotomy for the distribution of muscle unit fibers in a motor unit to exhibit clustering (figure 11). Again, the regenerating motor fibers demonstrated no specificity with regards to muscle fiber types reinnervated as previously shown (Gordon et al, 1988; Gillespie et al, 1987). I observed that the degenerating nerve sheath underwent a change in color from white to translucent after 2 weeks and subsequently became more fibrotic and smaller in size. Sometimes, the graft material was found enmeshed in network of connective tissue. However, at all times, taking the graft was accompanied by bleeding which tended to be profuse within the first 4 weeks but less so with increased duration of graft degeneration.

3.3. PROLONGED MUSCLE DENERVATION.

When freshly cut motor axons regenerated through a fresh autograft to innervate a long-term denervated tibialis anterior muscle, the recovery of muscle function and motor unit number was the poorest out of all the

three conditions. There was a progressive decline in muscle force (figure 12A) with increasing muscle denervation which was accompanied by a small but variable increase in motor unit force (figure 12B). The whole muscle twitch force was 1913 ± 115 mN when freshly denervated muscle (FDM) was reinnervated; and the force fell to 610 ± 167.5mN and 541.4 ± 331.8 mN with reinnervation of six and ten month long-term denervated muscle (LDM) respectively. The increase in motor unit force (20.5 ± 3.91mN in FDM , 36.5 ± 4.7 in 6 month LDM and 23.83 ± 6.96 mN in 10 month LDM) failed to compensate adequately for the dramatic fall in number of regenerating neurons which make functional muscle connections (figure 11C) in contrast to degenerated graft. The reinnervating motor units number was 110 ± 20 in FDM and this fell to 12 ± 3.6 in ten-month LDM.

Though the twitch force of reinnervated muscle units was normally distributed on logarithmic scales (figure 13), the reinnervated TA muscle remained atrophic as evident by figure 14(B) and 15(B & C), which showed muscle and muscle fiber with as little as 50% of normal CSA. The whole muscle CSA in FDM was 26.4 ± 1.9 mm^2 with progressive reduction to 10.1 ± 2.8 mm^2 in ten-month LDM. Further, with prolonged muscle denervation, there was a reduction in muscle fiber number implying loss of uninnervated fibers (fiber number in FDM was 9581 ± 514.5

and 6788 ± 1071.5 in ten-month LDM). This loss was not compensated for possibly because of exhaustibility of proliferative capacity of satellite cells pools in long-term atrophic muscle (Anzil & Wernig, 1989). Besides, long-term denervated muscle fibers did not recover their full size after reinnervation (figure 14C). The average fiber CSA declined continuously from 2752.3 ± 269.4 uM^2 in FDM to 1487.9 ± 382.7 uM^2 in ten-month LDM. Failure of full recovery in long-term denervated muscle (LDM) could also be due to a very slow process of recuperation from chronic lack of neural connection. This was exacerbated by reduced muscle fiber number and few regenerating motor neurons.

All these factors operating in long-term denervated muscle were also reflected in progressive loss of muscle weight with increasing denervation; and little recovery even after reinnervation (figure 16). Hence, it looks as if chronically denervated muscles are relatively refractory to neural influence which should reverse the atrophic changes.

3.4. SUMMARY.

Figure 17 summarizes the effect of delay contributed by the three components affecting regeneration: neuron, distal sheath and muscle, six months of delayed nerve repair. Effect of prolonged muscle denervation

contributed the most to poor muscle recovery (figure 17) after delayed repair by virtue of incomplete recovery from atrophy and decreased number of reinnervated motor units. On the contrary, cross-reinnervated TA muscle recovered fully after prolonged axotomy i.e muscle force recovered was similar to fresh axotomy (figure 17A) despite a small but insignificant fall in motor unit (figure 17C). The force recovery after repair with degenerated graft was quite similar to prolonged axotomy despite a more dramatic fall in motor unit number (figure 17C). It is not thus surprising to see in figure 17B that the regenerated units in the case of old graft repair branched the most to maintain the force and compensate for the decrease MUN. However, the sprouting capacity of the intramuscular motorneurons was not compromised in delayed nerve repair.

In conclusion, the lack of recovery of denervated muscle is the major determinant of poor functional recovery after delayed repair.

CHAPTER FOUR: DISCUSSION AND CONCLUSION.

The result of the present study clearly shows that prolonged muscle denervation is the major determinant of poor recovery of reinnervated muscles after delayed nerve repair, with lesser but significant roles being played by prolonged axotomy and progressive distal stump degeneration. Very few regenerating nerves make functional reconnection with long-term denervated muscle but these were able to branch inside the muscle to supply a maximum of 3-5 times normal number of muscle fibers. This branching is however not sufficient to reinnervate all the denervated muscles leading to over 40% reduction of reinnervated muscle fibers. On the contrary, cross-reinnervated TA muscles recovered fully after delayed axotomy before repair though this was associated with small but significant decline in number of regenerating nerves that make functional reconnection. A similarly good muscle force recovery was seen when freshly cut axons regenerated through a predegenerated autograft to reinnervate freshly denervated muscle despite a more drastic reduction in number of successfully regenerating motor units . The reduced MUN was fully compensated for by a tremendous increase in number of muscle fibers per motoneuron. Delayed nerve repair, however, does not compromise capacity of regenerating nerves to branch and

form enlarged motor units to compensate for fewer functional motor units.

4.1. RECOVERY AFTER IMMEDIATE NERVE REPAIR.

In order to examine the relative contribution of the deteriorating growth environment of the distal stump after delayed nerve repair, a peripheral nerve autograft was used to represent the environment. Thus the effect of progressive degeneration of nerve sheath on muscle recovery can be measured independent of prolonged axotomy or denervation. The introduction of the autograft however entails using two sutures when compared to direct nerve-nerve suture. Some workers have raised the possibility of an extra suture constituting a greater mechanical barrier to elongation past the suture line (Moy et al., 1988; Arshur et al., 1982). Thus, measurements of muscle and unit forces were compared after immediate nerve repair in three groups of rats: control (no suture), direct nerve-suture (one suture), and nerve-graft (two suture). There was no significant difference in muscle recovery in the three groups six months after the repair in agreement with the finding of Zhao et al. (1992).

We have calculated the motor unit number from measurement of muscle forces and sampling of 40-100% of motor units in the reinnervated muscles. Our technique of motor units counting has been validated in our laboratory

studies (Totosy de Zepetnek et al., 1992; Fu et al., 1993; You et al., 1994) and the numbers are in agreement with numbers determined using both horseradish peroxidase (HRP) and fluorochromes as retrograde labels (Swett et al, 1986; Crockett et. al, 1987).

4.2. PROLONGED AXOTOMY AND MUSCLE RECOVERY.

An injured nerve is able to survive, initiate sprouting, elongate and make functional reconnection with its target. With delayed nerve repair and prolonged axotomy, the axotomized neurons must continue to express adequate level of growth-associated proteins for optimal regeneration. In this study, nerve repair as late as six months after tibial nerve axotomy resulted in good force recovery in cross-reinnervated TA muscle in agreement with maintained regenerative capacity obtained physiologically by Holmes and Young (1945) and regeneration-associated gene (RAG) measurement by Tetzlaff et al (1992). The increase in number of muscle fibers reinnervated per motoneuron with delayed repair fully compensates for the small reduction in number of motor units in the reinnervated muscle.

The reduced number of motor units after prolonged axotomy could be due to death of motoneurons or reduced capacity of long-term axotomized neurons to elongate over distance and functionally reconnect with muscle. Survival

of motoneurons in adult after axotomy (both short and long-term) has been documented by several workers (Carlsson et al, 1979; Gordon et, al 1991; Vanden Noven et al, 1993; Schmalbruch, 1984) except in cases of facial and hypoglossal nerve section (Arvidsson and Aldskogius, 1982; Yu, 1988). Hence, the decline in reinnervating neurons cannot be attributed to neuronal death though this can be tested experimentally by using retrograde dye labelling and counting of neuron cell bodies.

The final possibility is the reduced capacity of the chronically axotomised neurons to maintain growth-associated events over time. Some of the neuron growth efforts might have been dissipated in neuroma formation and retrograde elongation during the period of chronic axotomy. Further, on second axotomy needed to freshen the distal end of proximal neuron there is another wave of sprouting and some which will later be withdrawn. This second freshening axotomy may also trigger another wave of Schwann cell proliferation and migration which has been reported to be greater 2-3 weeks after first severance (Holmes and Young, 1942). It is conceivable that the capacity of Schwann cells to proliferate slowly declines with longer time when the cellular outgrowth may be insufficient to bring about effective union of nerve ends. All these events make a tremendous metabolic demand on the neurons which by dissipating its resources over

several pathway ultimately diminish their ability to elongate and make functional muscle reconnection.

The capacity of reinnervating neurons to branch inside the muscle is not compromised by prolonged axotomy. The motor unit size as measured by force generated by muscle fibers in a motor unit, shows a rising trend in parallel with change in motor unit numbers. This implies optimal response to trophic and substrate influence operating at the level of intramuscular nerve sheath. The reinnervating axons presumably exhibit the appropriate temporal and spatial combination of surface adhesion molecules, such as N-CAM, L1 and integrin, which interact with the muscle basal lamina and resident non-muscle cells. The maintained branching capability of the motor neurons fully compensate for the reduced motor unit to give a good force recovery.

The functional recovery is also reflected in excellent recovery of CSA, muscle weight and muscle fiber number and size. However, the distribution of muscle units is clustered in contrast to the mosaic pattern seen in normal muscle (Kugelberg et al, 1970; Totosy de Zepetnek et al, 1992). This clustering of muscle unit fibers is an indication of failure of regenerating motor axons to reinnervate their original muscle fibers. For instance, axons that normally innervate slow-twitch

muscle fibers show no preference for these fibers and will innervate fast-twitch fibers alike (Gillespie et al, 1986; Gordon, 1988).

4.3. PROLONGED GRAFT DEGENERATION: EFFECT ON FUNCTIONAL RECOVERY.

The results of this study show that when a nerve autograft is predegenerated for up to 1 year prior to its insertion into a nerve defect, fewer nerves regenerate through and make functional reconnection. It is now known from several experimental studies that the Schwann cell and its basal lamina are crucial components in the environment through which regenerating axons grew to reach their peripheral sensory and motor targets (Lundborg, 1989; Ramon Y Cajal, 1928; Varon and Bunge, 1978).

The progressive decline in quality of the growth environment with increasing period of distal stump degeneration can reduce the number of regenerating axons and functional recovery. During chronic degeneration of peripheral stump, previous in vivo studies have demonstrated discontinuity and partial disappearance of Schwann cell basement membrane (SCBM), atrophy of the Schwann cell columns and a decrease in laminin immunoreactivity associated with them (Salonen et al, 1987; Giannini and Dyck, 1990; Weinberg and Spencer,

1978). Similar results have been obtained in experimental nerve grafts (Gulati and Zalewski, 1985). In addition, there is increasing collagenization and shrinkage of endoneurial tube leading to a reduction in tube diameter (Holmes and Young, 1942). The compromised caliber of the endoneurial sheath will constitute a mechanical barrier to the advancing axons. Besides, it is not known whether resident Schwann cells in long-term denervated stump continue to provide optimal trophic support for regenerating neurons. However, the migration of Schwann cells from proximal stump might partly compensate for reduced number of Schwann cells in the distal stump. Nonetheless, the fragmented basal lamina scaffold might not support optimal migration of such Schwann cell over a long distance. Hence the dismal number of regenerating neurons with prolonged degeneration could be due to the mechanical barrier of the fibrotic stump or lack of trophic support of non-neural cell and their basal lamina or combination of both. Furthermore, there is more possibility for aberrant entrance of proximal regenerating axons into inappropriate distal endoneurial tube because of gross mismatch attributed to shrunken distal stump.

Some or all of these factors may have acted together in our study, to cause a significant decrease in number of regenerating axons that make functional connection.

The high variability seen in the outcome of recovery in this study reflect the multitude of factors that bear on the elongating motor axons. Nonetheless, the few neurons that grow into the muscle branch profusely to innervate more muscle fibers from previous motor units. Hence, the muscle force recovery is comparable to normal.

Repair of damaged peripheral nerves with autologous grafts is a routine neurosurgical procedure (see Sunderland, 1978). The possibility of using stored nerve allografts has been considered for elective peripheral nerve allograft reconstructive procedure (Mackinon et al, 1992.,Sunderland, 1978; Kernset at al, 1993) particularly with injury/disease that destroyed a considerably long nerve segment. The repair of large gaps with nerve graft presents a challenging problem for the clinicians for several reasons: suture line barrier, poor graft revascularization, axonal misrouting and insufficient donor nerve material. Hence collecting and storing donated nerve tissue for later use might be one way of reducing the problem of inadequate supply of nerve material. However, for such stored graft to be effective in supporting nerve regeneration, the compromising effect of deteriorating nerve sheath environment observed in our study must be considered. The growth of axons could be enhanced in such nerve graft by administration of appropriate trophic factors or by the use of silicon

chambers filled with laminin, fibronectin and relevant growth factors (Raivich and Kreutzberg,1993, Kanje et al, 1989; Bailey et al, 1993). However, the few studies available on this method (Bailey et al, 1993; Wooley et al, 1990; Williams et al, 1983) failed to measure the number of neurons that make functional reconnection: the ultimate determinant of clinical recovery. Hence, in order to determine the feasibility of storing peripheral nerves for later use as allografts to repair nerve transection injuries, there is a need to examine not only the number of axonal sprouts but also the ability of the regenerating neurites to make functional reconnection.

4.4. PROLONGED MUSCLE DENERVATION: EFFECT ON FUNCTIONAL RECOVERY.

The recovery of muscle force and weight depends both on the number and size of the muscle fibers; and these in turn depend on how many nerves reinnervate the muscles, the number of muscle fibers reinnervated per regenerated nerve and the reversal of denervation atrophy in these reinnervated muscle fibers. Thus the extremely poor recovery of muscle function in this study reflect changes at various levels.

With prolonged denervation of muscle, the intramuscular nerve sheaths progressively deteriorate in a manner similar to degenerating distal nerve stump.

Also the proportion of old end-plates which becomes reinnervated is progressively reduced (Gutmann & Young, 1944). Further, it is conceivable that long-term denervated muscle may not maintain a level of basal lamina, extracellular matrix molecules and non-muscle cells adequate for intramuscular elengation of reinnervating axons (Booth et al, 1990). For instance Gutmann and Young (1944) reported that most of intramuscular nerve fibers escaped from the shrunken sheath and regenerated on surface of muscle fibers. It is worth noting that the surface of atrophic muscles may not adequately support the regenerating neurons. The axons also face the hurdle of increased connective tissue in the thickened perimysium and epimysium (Sunderland, 1978)

The finding of few regenerating neurons in muscles which were denervated for as long as one year is a reflection of the above-mentioned obstacles to nerve elongation. The branching ability of these few neurons does not seem to be affected but there is a 3 - 5 fold limit to their capacity to enlarge their motor units. Hence, the neurons could not completely innervate all the available muscle fibers belonging to other axons. It is thus not surprising that the muscle force recovery is less than 25% of normal.

The poor recovery is further exacerbated by severe

muscle fiber loss in the denervated muscle. A similar event was observed by Gutmann and Young in 1944 when they stated that "many muscle fibers had disappeared altogether and others were unable to respond even though nerve fibres branched among them". This loss is shown in the decline in muscle weight and size recovery in the present study. Also, the reinnervated muscles may not recover fully from the previous atrophic changes because the muscle fibers size, as calculated by fiber density, is still below normal even up to 1 year after reinnervation. It is also possible that recovery of muscles reinnervated after prolonged denervation is a very slow process (Irintchev et al, 1990).

The drastic reduction in muscle fibre numbers after denervation has been well documented by previous studies (Anzel and Wernig, 1989; Gutman and Young, 1944; Gutmann and Zelena, 1962). However, with short-term muscle denervation, satellite cells are induced to proliferate and form new muscle fiber (Ontel, 1974). With prolonged muscle denervation before reinnervation, the proliferative capacity of the satellite cells might be exhausted as suggested by Anzil and Wernig (1989). This muscle loss may be exacerbated in presence of ischemia, immobility and direct injury to the muscles. Hence, it is important in any in vivo experiment that investigates muscle denervation to avoid complete restriction of

movement of the animal. For instance, this study ensures the free movement of the rats by specifically denervating only the muscles of interest. An immobilized lower limbs suffer from compromised blood supply, stasis and edema; and these in turn will affect muscle nutrition and modify muscle recovery measurement (Savolainen et al, 1988; Sunderland, 1978).

Finally, lack of specificity of axonal guidance operating at the levels of suture line, degenerating distal endoneural tubes and intramuscular nerve sheath may further jeopardize functional recovery. For instance, it is very possible in this study that some motor axons are misrouted into inappropriate sensory endoneurial tube. No functional motor units will be formed in such situation and several studies have described this phenomenon as a cause of poor functional recovery (Sunderland, 1978; Millesi,1988; Thomas et al, 1987; Brushardt and Mesulam, 1980). The erroneous cross-shunting of axons is further aided by production of multiple offshoots from surviving axons. Such multiple sprouts from either a single or different axons may enter the same tube simultaneously with eventual survival of only one neurite (Sunderland, 1978). If the axons of an originally short nerve fibre enter an endoneurial tube originally occupied by a long fiber, it will grow the full length of the tube. It is thus conceivable that the

neuron cell body may be unable to satisfy these additional needs. Another consequence of aberrant innervation on functional recovery is ineffective contraction that will be produced if say a flexor muscle is innervated by extensor motoneurons.

4.5. CONCLUSION.

The major findings of this study are that prolonged muscle denervation is the major determinant of poor recovery of reinnervated muscles after delayed nerve repair. Long-term axotomy and progressive distal stump degeneration play lesser but important roles in the recovery outcome. However, delayed nerve repair does not compromise the ability of regenerating nerves to branch and innervate more muscle fibers than normal. This branching serves as a compensatory mechanism for the fewer functional units. Progressive degenerative changes in long term denervated distal stump drastically reduce the number of regenerating axons but the few axons fully compensates for this by innervating more fibers to give a good force recovery. The long-term axotomized neurons can still initiate, maintain regeneration-associated events and make functional reconnections. Therefore, the poor recovery in reinnervated muscle after delayed nerve repair is mainly a reflection of incomplete reversal of denervation atrophy caused by long-term denervation and not long-term axotomy or atrophic distal stump.

5. FIGURES AND LEGENDS.

Error bars represent standard error of the mean in all the figures where data are plotted as arithmetic means.

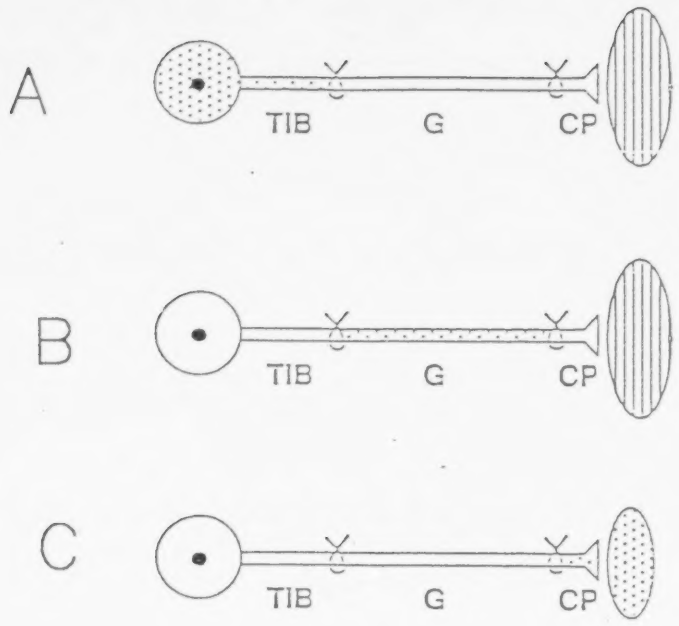


Figure 1. Schematic diagram of surgical paradigms. (A): Long-term axotomized tibial (TIB) neurons regenerated via fresh contralateral common peroneal (CP) autograft to innervate freshly denervated Tibialis Anterior (TA) muscle. (B): Contralateral CP autograft was sutured to freshly cut tibial nerve which subsequently innervated freshly denervated TA muscle. (C): Long-term denervated TA muscle was innervated by freshly axotomized tibial nerves which grew via a fresh contralateral CP autograft.

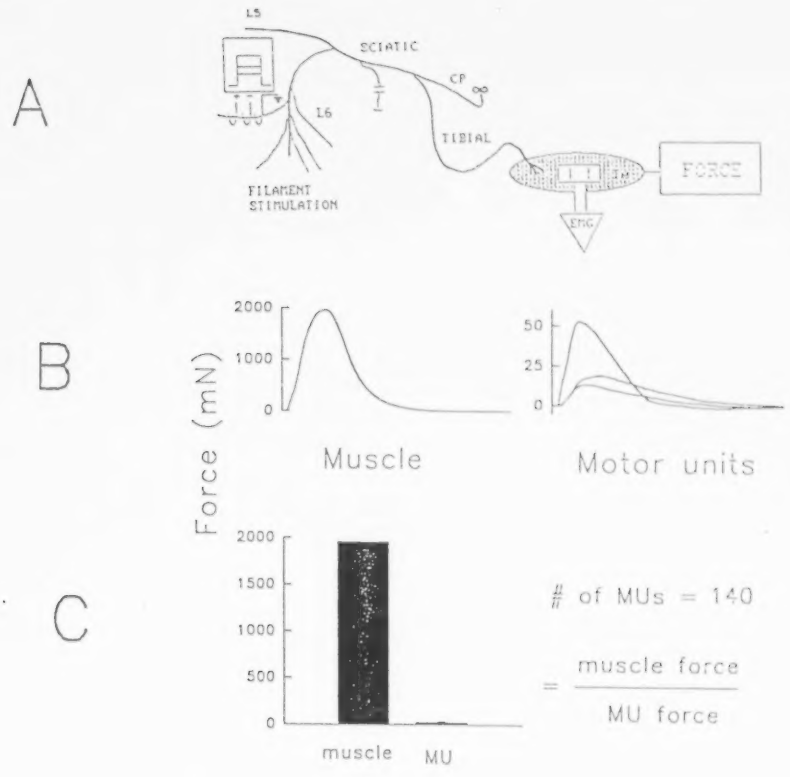


Figure 2: Motor unit recording. (A): Diagrammatic illustration of denervation of all muscles in the rat hindlimbs other than TA muscle, stimulation of isolated ventral root filaments and recording of muscle force and EMG. (B): All-or-none increments in force are recorded from TA muscle in response to incremental increases in stimulus voltage to the ventral root filaments.
Indirect measures of motor unit size are made from the force increments and by subtraction. (C): Motor unit counts are made by dividing the whole muscle twitch force (in response to stimulation of the muscle nerve) by the mean MU force (from 40-100% of the population).

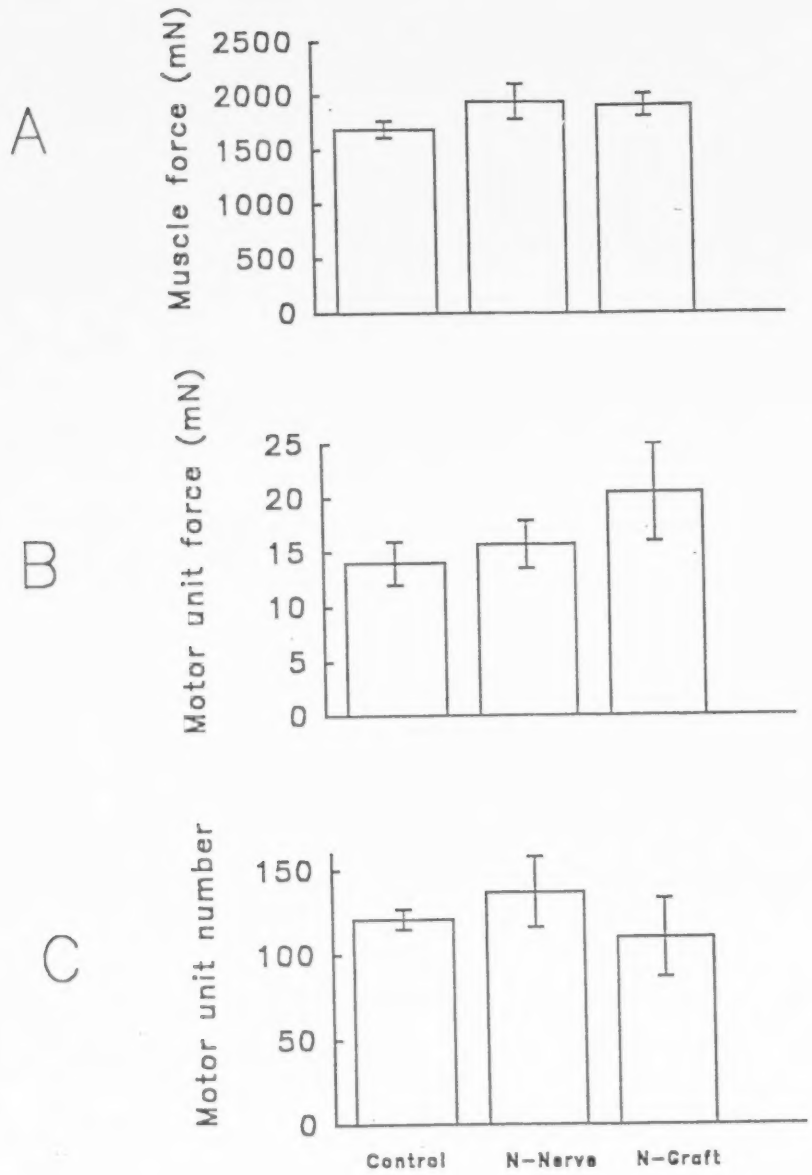


Figure 3. Comparison of twitch muscle force recovery in control rats and after immediate repair in conditions of one suture (nerve-nerve union) and two sutures (nerve-graft union). The muscle and motor unit force and motor unit number recovery were not significantly different in the three groups.

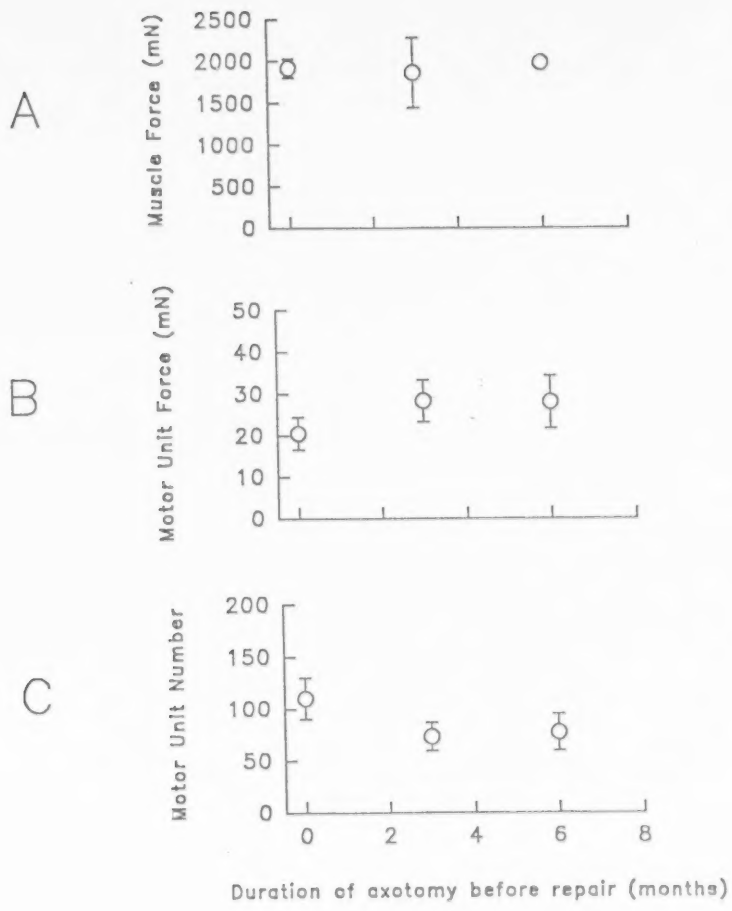


Figure 4. Effect of prolonged tibial (TIB) nerve axotomy on the muscle force recovery and number of TIB motor nerves which make functional nerve-muscle connections. There was good muscle force recovery after six months of axotomy prior to repair (A). There was small but significant increase in motor unit size (B) which fully compensated for the gradual decline observed in reinnnervating motor neurons (C).

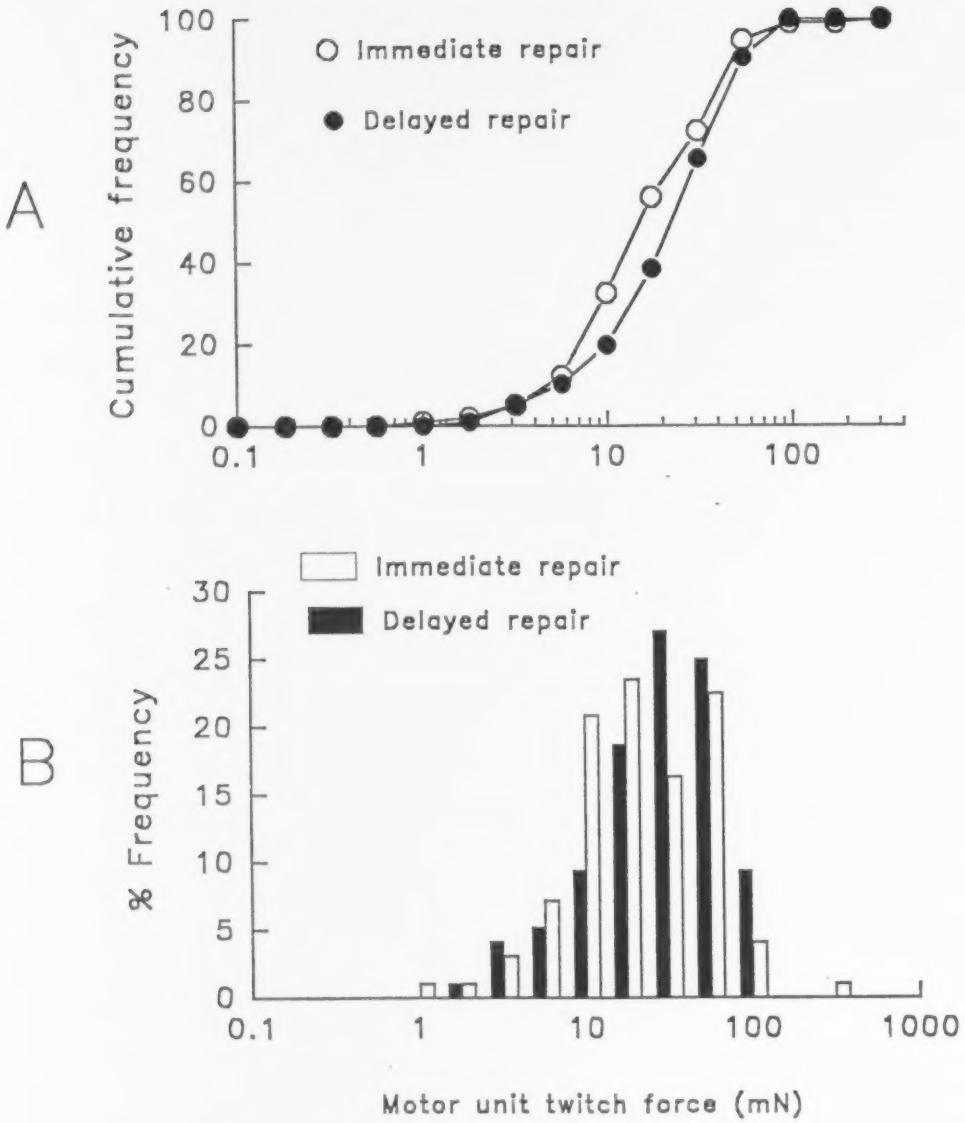


Figure 5. Motor unit twitch force distribution after prolonged axotomy. The reinnervated motor unit population after delayed repair is shifted slightly but not significantly to the right along the force axis indicating an increased motor unit size which accompanied the fall in motor unit number.

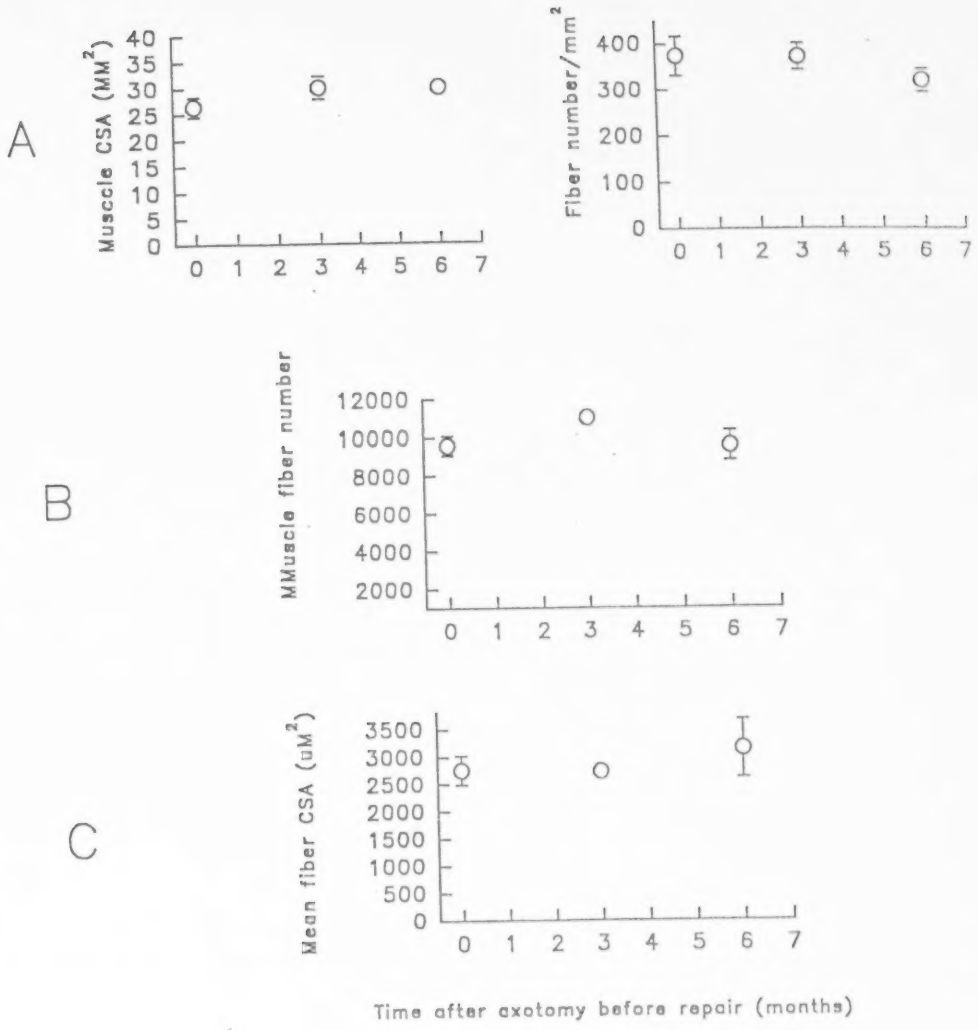


Figure 6. Effect of prolonged axotomy before repair on recovery of reinnervated muscle CSA, muscle fiber number and size. There was no significant change in reinnervated muscle CSA, muscle fiber number and size whether nerve repair is immediate or delayed.

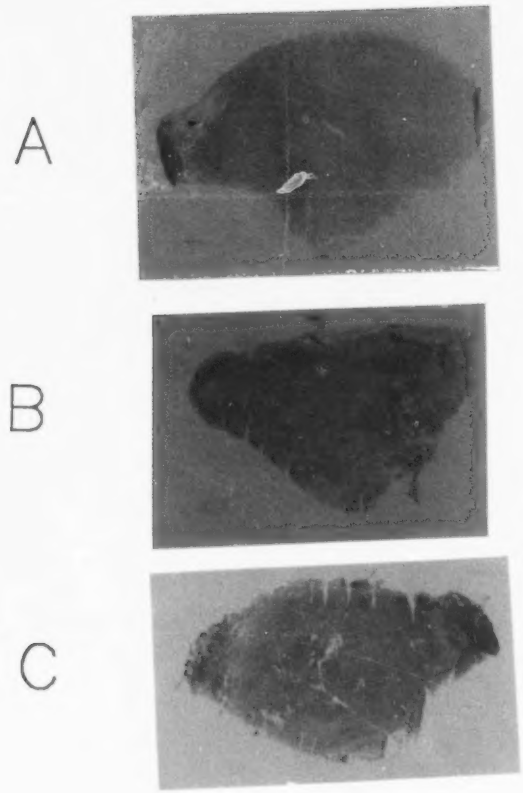
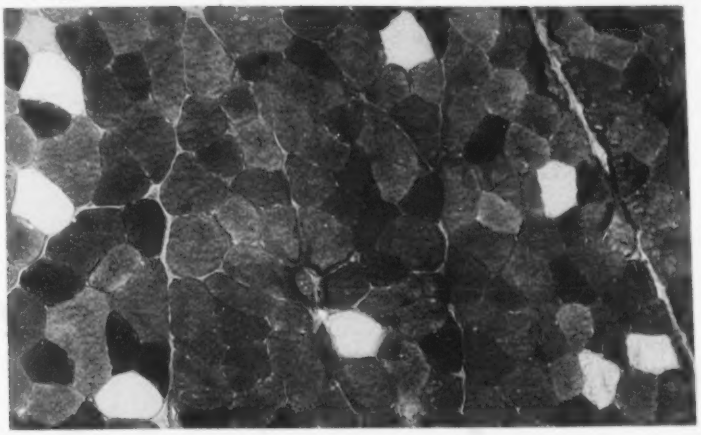


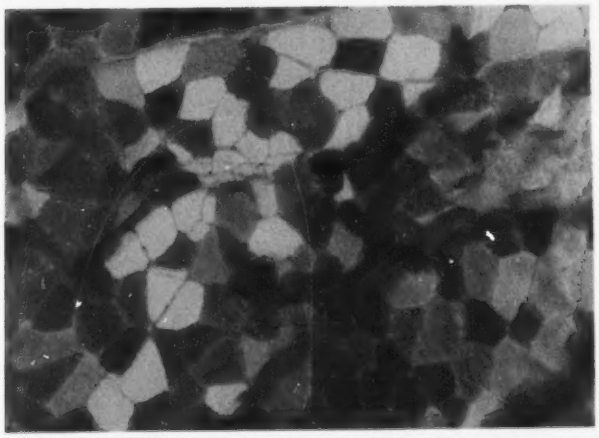
Figure 7. Comparison of cross-sectional area of whole muscle and fiber distribution of glycogen-depleted muscle units in normal (A) and TA muscles cross-innervated by freshly cut motor neurons (B) or six-month axotomized neuron (C) via a CP autograft. The CSA of reinnervated muscle (B & C; top figure) is similar to control (A; top figure) but there was clumping of glycogen-depleted muscle fibers in a single unit (white area) in the reinnervated muscle (B & C; figure on page 68), in contrast to the mosaic pattern of normal muscle fiber units (A; page 68).

High power: 6.3X

A



B



C

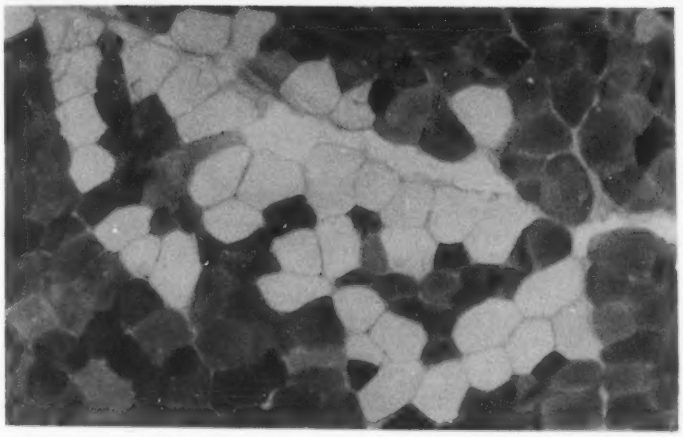


Figure 7. (continued): PAS-stained section of glycogen-depleted muscle unit fibers

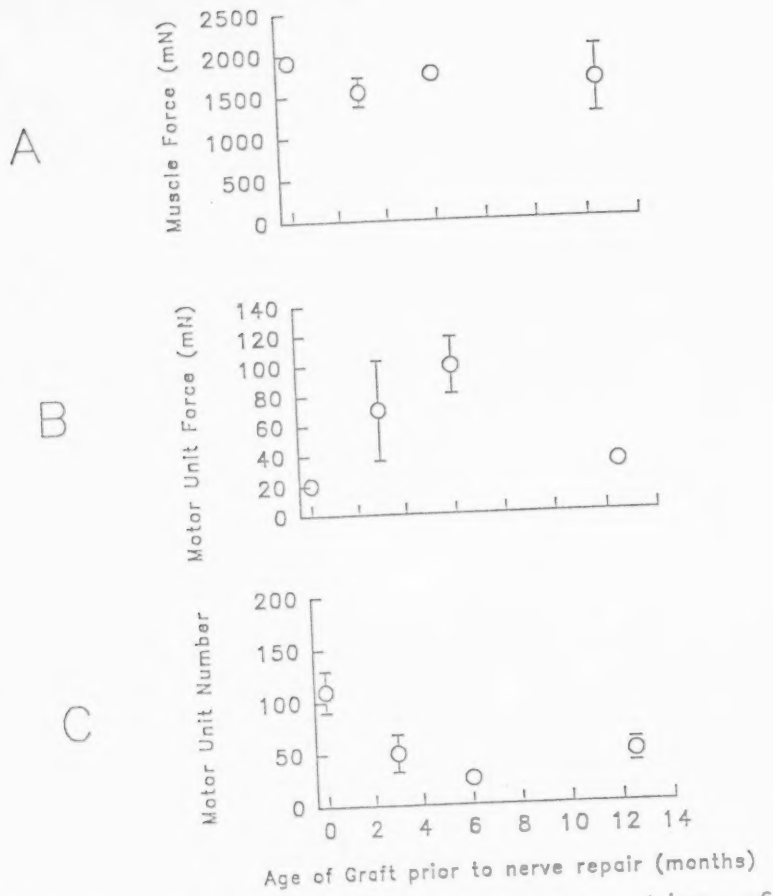


Figure 8. Effect of prolonged degeneration of distal nerve stump on muscle force recovery and number of tibial motor neurons which make functional nerve-muscle connections. Muscle force recovery was excellent even when tibial motor nerves regenerate through a one-year predegenerated nerve sheath. There was dramatic increase in mean motor unit size (up to 6 month-old graft) which fully compensated for the dramatic fall in reinnervating motor unit number. The motor unit force value at 12 months is for one rat and may not sufficiently explain the muscle force recovery.

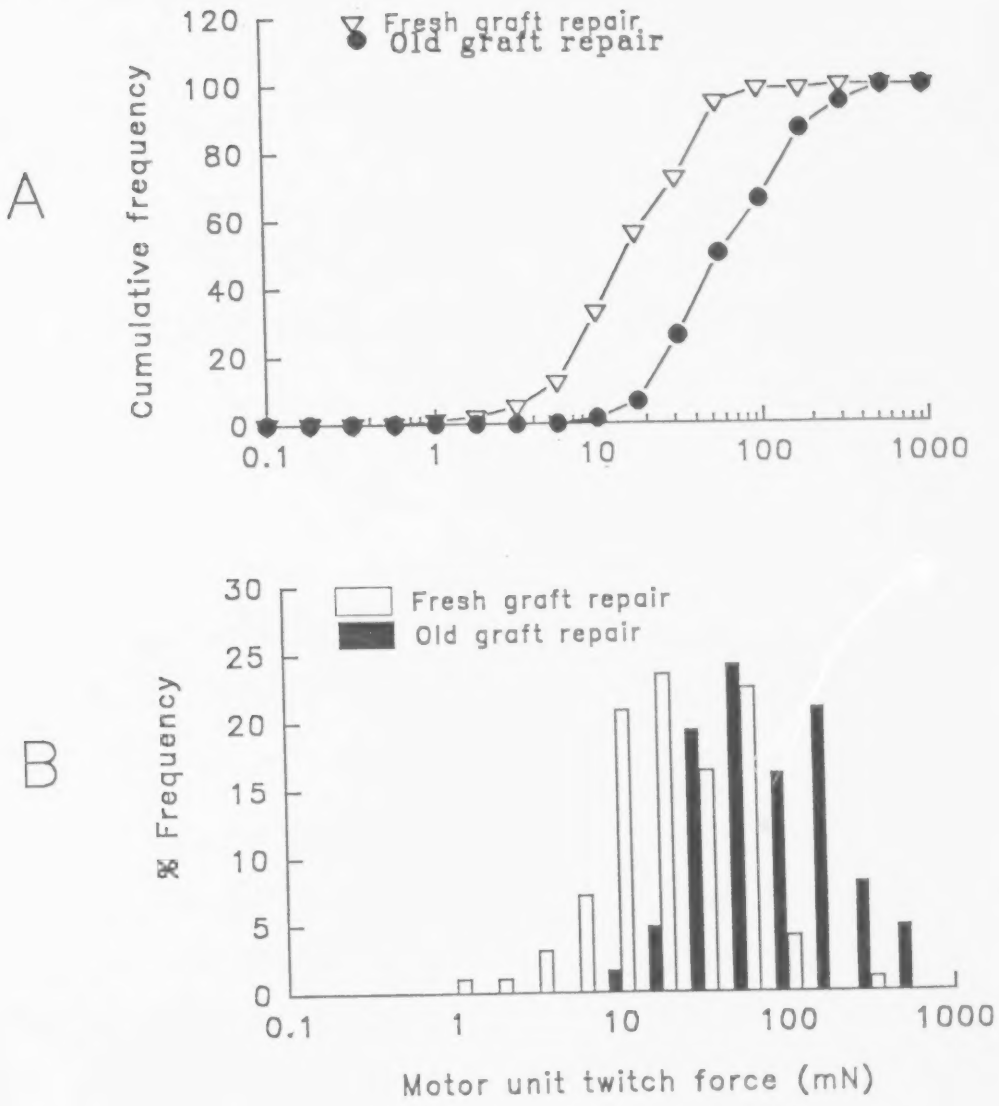


Figure 9. Motor unit size distribution after tibial nerves regenerated through long-term denervated nerve sheath. The reinnervated unit population distribution is normal though shifted significantly to the right due to increased branching of the fewer reinnervating neurons.

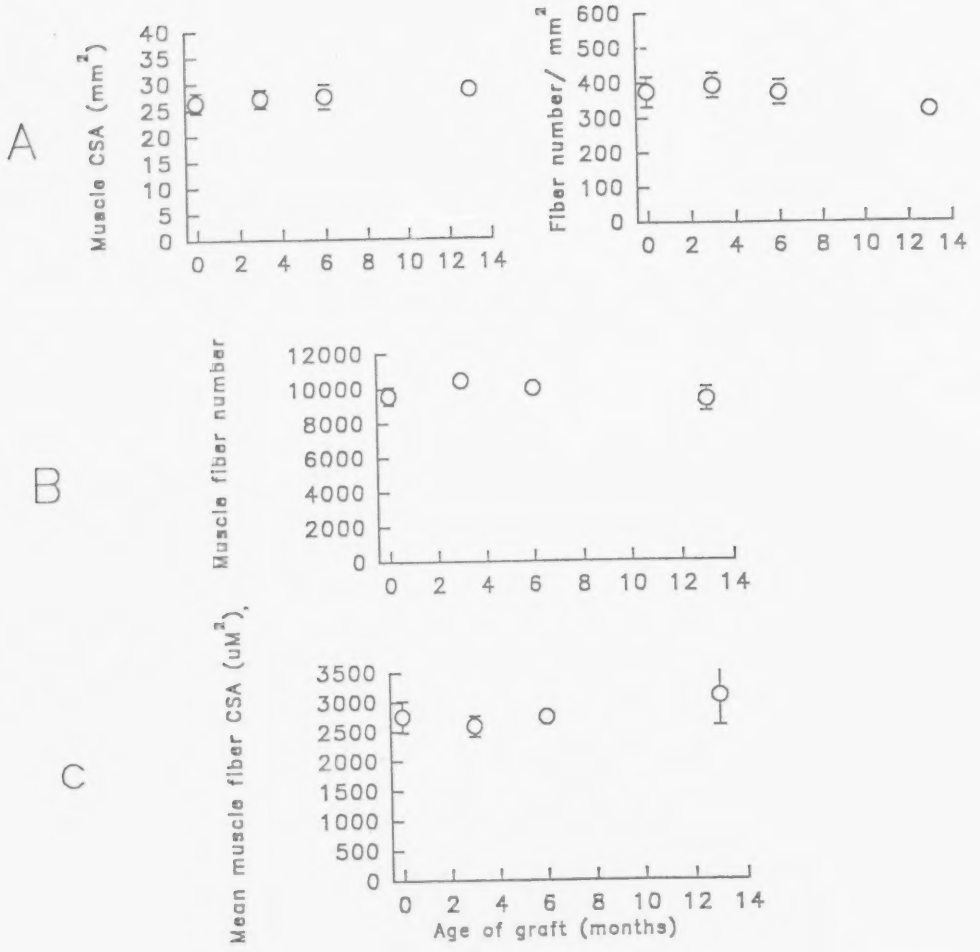


Figure 10. Effect of prolonged distal nerve sheath denervation before nerve repair on recovery of reinnervated muscle CSA, muscle fiber number and size. Whether nerve repair was done with fresh nerve sheath or predegenerated one, the recovery of reinnervated muscle CSA, fiber number and size is similarly good.

A

B

C

Figure 11. Comparison of CSA of whole muscle and fiber distribution of glycogen-depleted muscle units in normal (A) and TA muscles cross-innervated by tibial neurons regenerating via a fresh graft (b) or one-year predegenerated nerve sheath (C). Fresh or degenerated nerve graft results in similar muscle size recovery which was as good as normal TA (A, B & C; upper figure). However, the reinnervated TA muscle showed a clumping of glycogen-depleted muscle fiber unit as opposed to random pattern seen in normal TA muscle units (A,B & C; figure on page 73).

High power: 6.3X

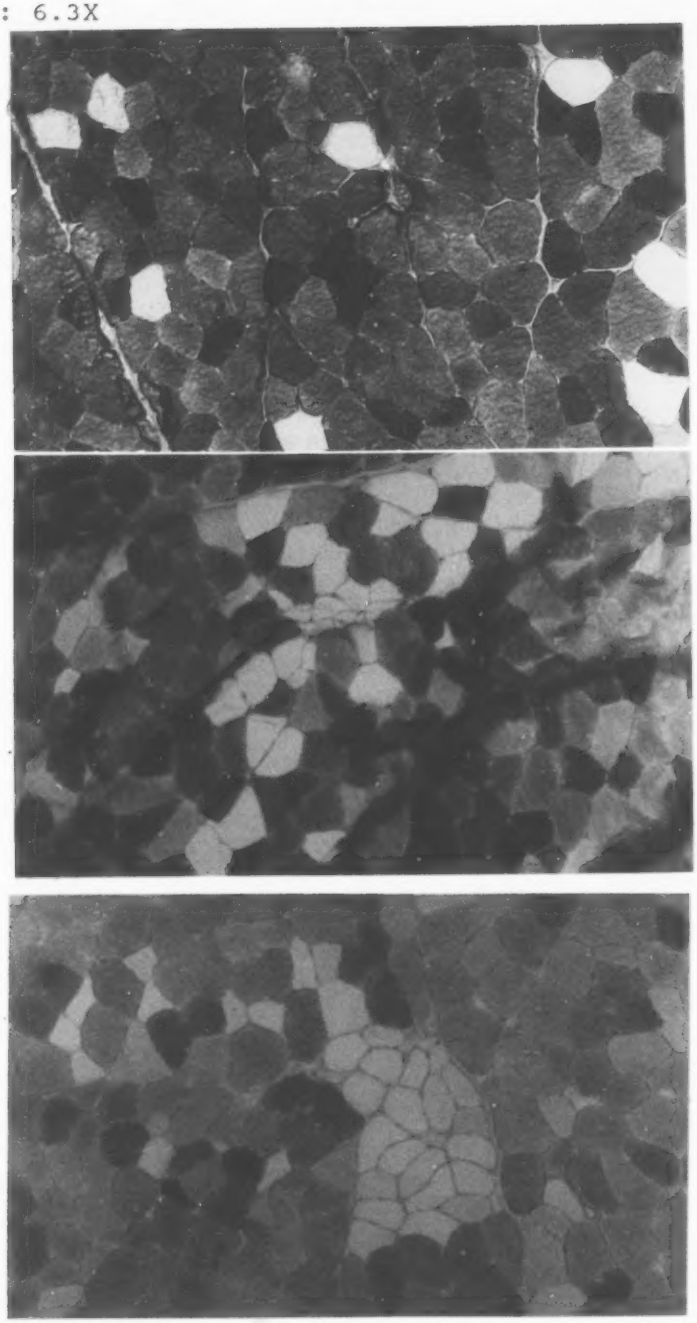


Figure 11. (continued): PAS-stained section of glycogen-depleted muscle unit fibers.

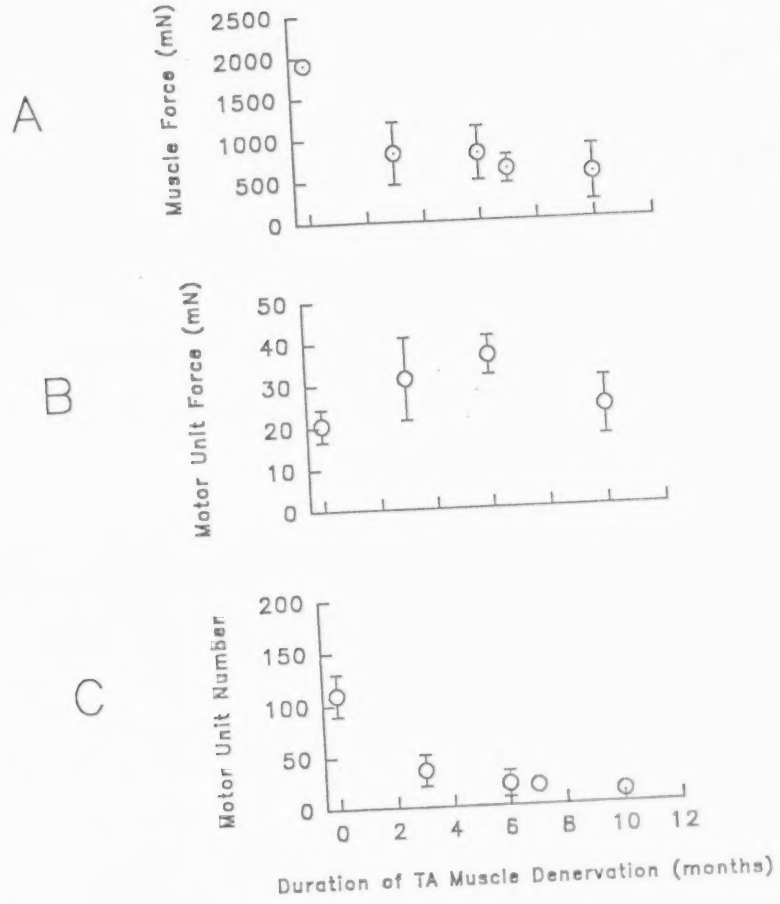


Figure 12. Effect of prolonged TA muscle denervation on muscle force recovery and number of reinnervating tibial motor neurons. The progressive fall in muscle force (A) with increasing muscle denervation was associated with a small but variable increase in motor unit force (B) which did not fully compensate for the dramatic decline in number of regenerating motor neurons (C).

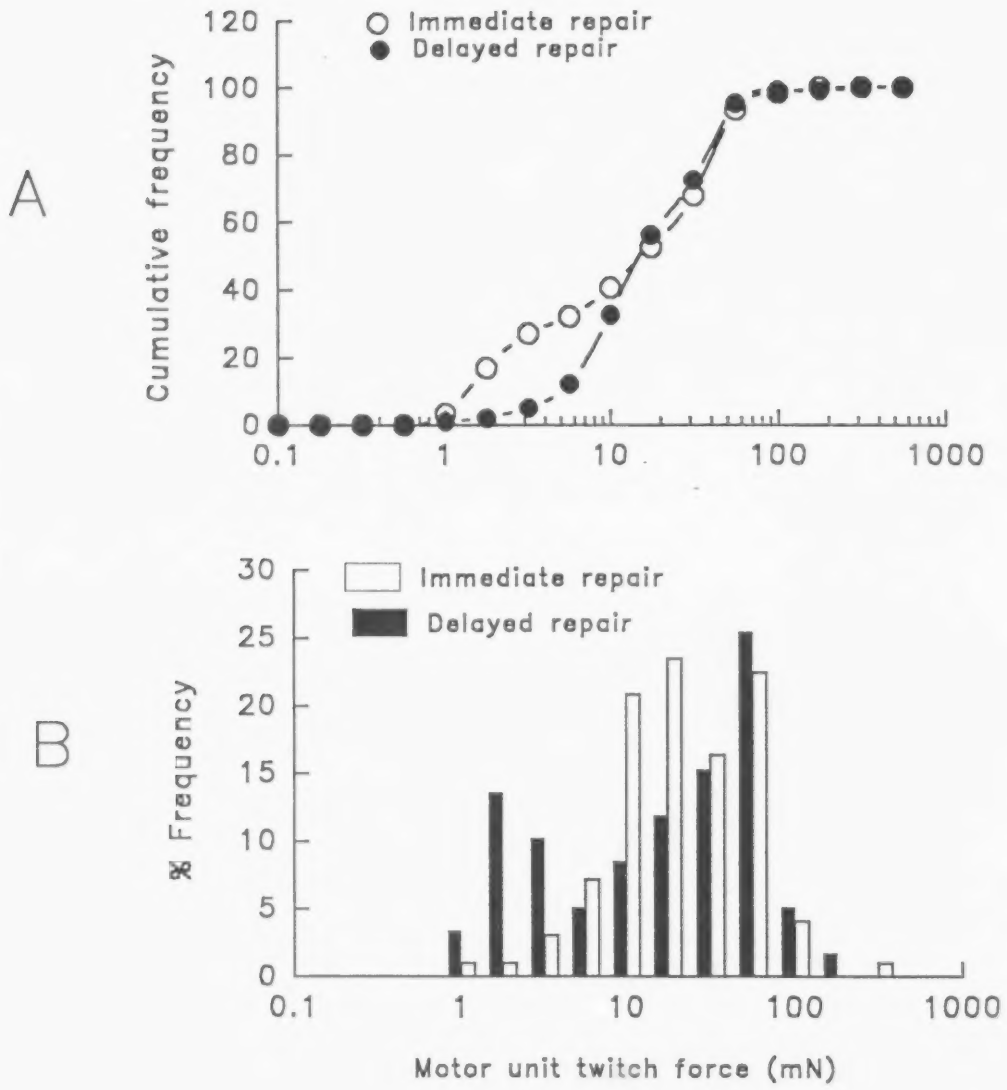


Figure 13. Motor unit size distribution after reinnervation of long-term denervated TA muscles. The reinnervated muscle unit population distribution was not significantly different from normal despite a slight shift to the right due to increased branching of the very few reinnervating motor units.

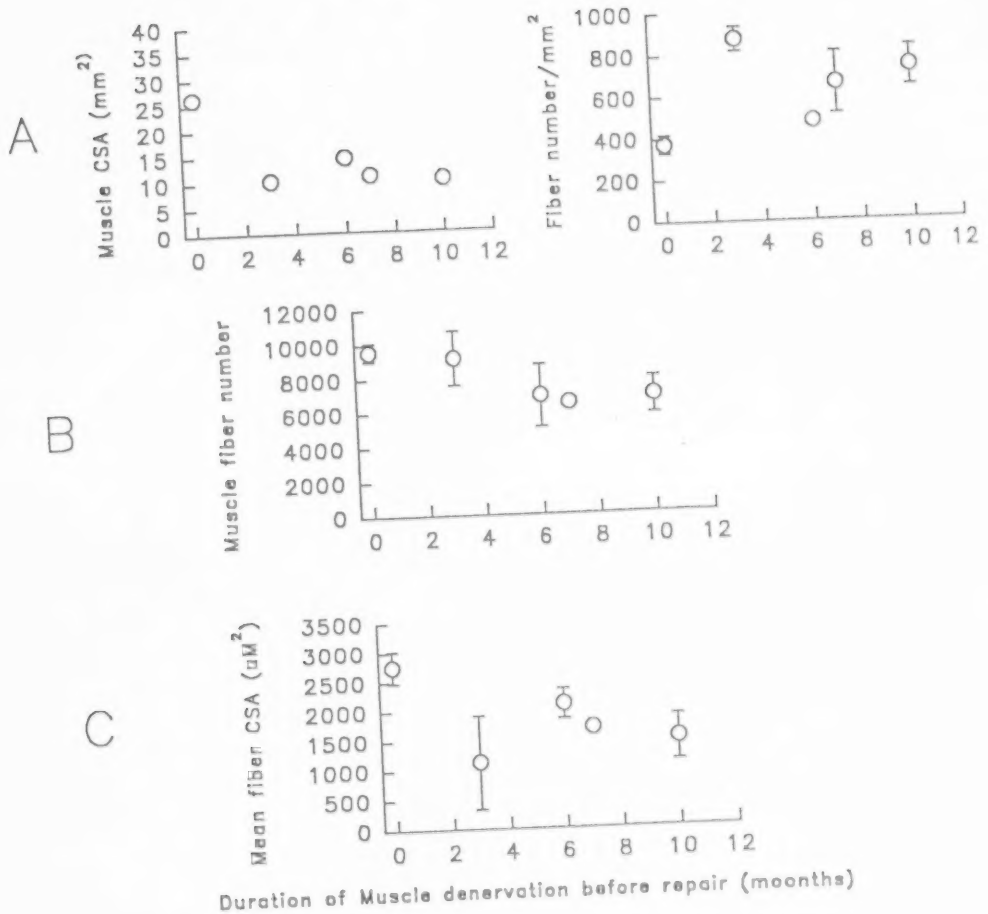
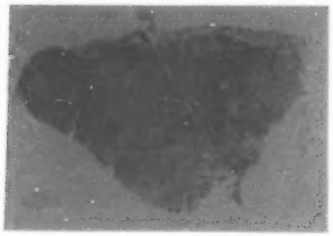


Figure 14: Reinnervation of long-term denervated TA muscle: effect on muscle CSA, muscle fiber number and size. With increasing duration of muscle denervation, there was a progressively severe reduction of muscle CSA, muscle fiber number and fiber size of the reinnervated muscle. Chronically denervated muscle did not recover fully from atrophic changes.

A

B



C

Figure 15. Comparison of CSA of whole muscle and fiber distribution of glycogen-depleted muscle units in normal (A), freshly denervated (B) and long-term denervated TA muscle (C). The reinnervation of long-term denervated TA muscle resulted in reduction of whole muscle size when compared to normal TA muscle and freshly denervated muscle (A, B & C; upper figure). The figure on page 78 shows clustering of glycogen-depleted muscle unit fibers (the white fibers) in the reinnervated muscles (B & C) in contrast to control TA muscle .

High power: 6.3X

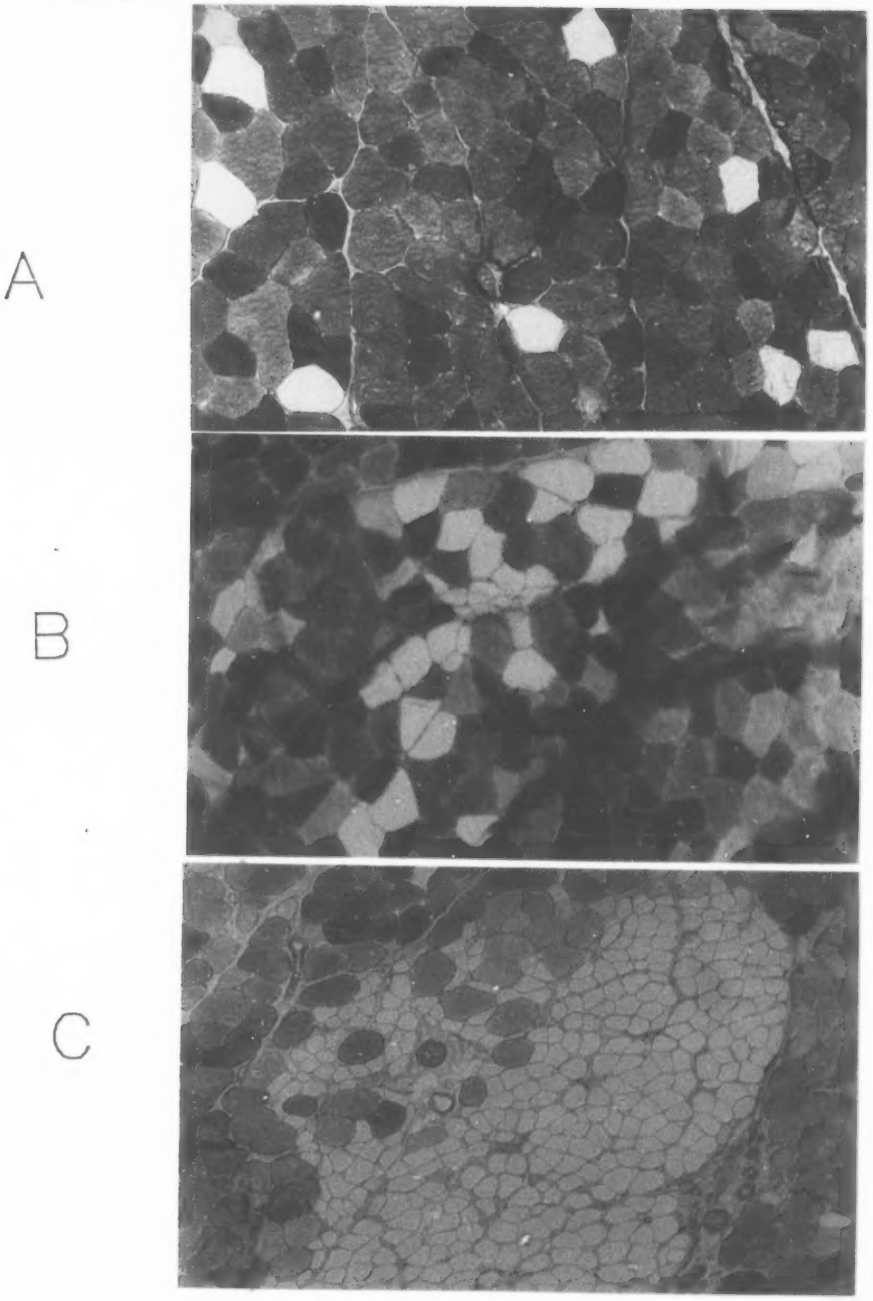


Figure 15. (continued): PAS-stained section of glycogen-depleted muscle unit fibers.

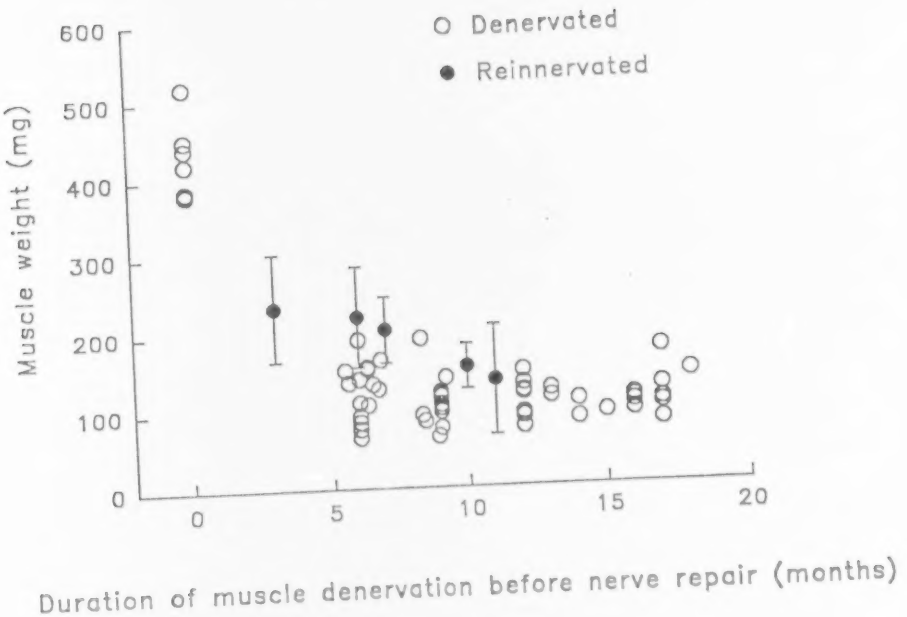


Figure 16. Muscle weight recovery after reinnervation of long-term denervated TA muscle. There was a progressive loss of muscle weight with increasing duration of muscle denervation prior to nerve repair.

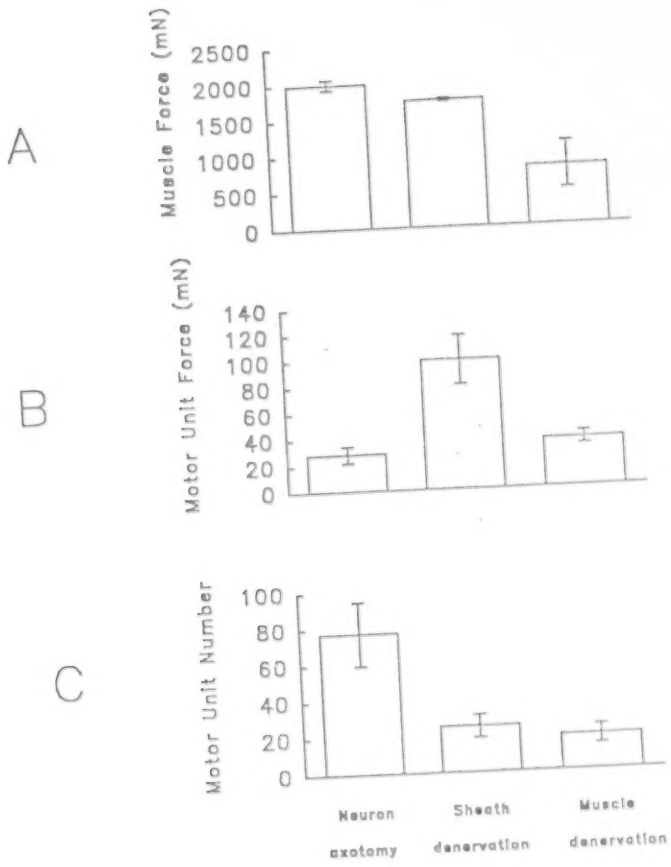


Figure 17. Summary of data on regeneration outcome after delayed nerve repair. Effect of prolonged muscle denervation contributes the most to poor functional outcome after delayed nerve repair, as depicted by the poor force recovery (A) and moderately increased motor unit force (B) which did not fully compensate for the few reinnervating motor neurons (C) when compared to prolonged axotomy or nerve sheath denervation. Prolonged axotomy is the least affected by delayed nerve repair.

6. REFERENCES

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